# GENETIC MANIPULATION OF FAT IN TRANSGENIC MICE

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To my lovely wife and son.

I throughout this time, and Bruce Whitelaw for many helpful and stimulating discussions.

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I hereby declare that the work presented in this thesis is the product of my own efforts, and has not been submitted in any previous application for another degree. The work on which it is based is my own except where stated in the text or in the acknowledgements.

Ricardo Felmer

The discovery of regulatory elements that can direct tissue specific expression to adipocytes has made possible experiments to study the function of endogenous genes in fat metabolism and to assess the effect of these genes in other metabolic pathways. It has also opened up the possibility of regulating the fatness of laboratory and domestic animals by directing targeted expression of cytotoxic genes to this tissue.

The present dissertation describes the use of a novel system to achieve specific cell ablation in fat tissue. The method is based on the use of *E.coli* nitroreductase (NTR) enzyme that activates certain nitro compounds into cytotoxic DNA interstrand cross-linking agents. This system was assessed first *in vitro*, in a preadipocyte cell line (3T3L1). Clones of cells that expressed NTR were successfully killed after treatment with CB1954. It was confirmed that the mechanism of cell killing involved is apoptosis and the presence of a cell-permeable metabolite that is released to the medium triggering a bystander effect was observed.

This prodrug system was also assessed *in vivo*, for which transgenic mice were generated expressing NTR specifically in adipose tissue under the control of the aP2 promoter. Upon CB1954 treatment, transgenic mice showed extensive cell depletion in different fat deposits, which was directly correlated to both the dose of prodrug and the levels of NTR expressed. The present model provides a new inducible approach to manipulate the number of adipocytes at different stages of the mouse development and provides a new system for the study of fat metabolism especially in abnormal conditions such as obesity and its modulation through the manipulation of the target cell population.

Also reported are preliminary experiments to assess a novel system of ablation mediated by the murine adapter molecule RAIDD. Stable cell lines were generated to overexpress RAIDD after differentiation. A range of phenotypes was observed with these clones from a complete blockage of the differentiation to the killing of cells that escape the blockage. The present results suggest a new developmental role for this gene and strongly encourage further experimentation to confirm this effect in an experimental animal model.

**Chapter 1**



**Chapter 3**



**Chapter 4**

| | |
|---|---|
| Amp | Ampicillin |
| BAT | Brown adipose tissue |
| bp | Base pair (s) |
| °C | Degrees Celsius |
| cDNA | Complementary DNA |
| cpm | Counts per minute |
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphophate |
| $ddH_2O$ | Double distilled water |
| dGTP | 2'-deoxyguanosine 5'triphosphate |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNase | Deoxyribonuclease |
| dNTP(s) | Deoxynucleotide triphophate |
| *E.coli* | Escherichia coli |
| EDTA | Ethylenediamine-tetra-acetic acid |
| EGTA | Ethyleneglycol-bis(β- aminoethyleter)-N,N'-tetra-acetic acid |
| EtBr | Ethidium bromide |
| FCS | Fetal calf serum |
| g | Gram |
| x g | Gravitational force |
| HCl | Hydrochloric acid |
| HEPES | N-2-hydroethylpiperazine-N'-2-ethane sulfonic acid |
| hr(s) | Hour(s) |
| kb | Kilobase |
| KCl | Potasium chloride |
| kD | Kilodaltons |
| l | Litre(s) |
| LB | Luria broth |
| M | Molar |
| µg | Microgram |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| MgSO4 | Magnesium sulphate |
| min(s) | Minute(s) |
| µl | Microlitre(s) |
| ml | Millilitres |
| mM | Millimolar |
| MOPS | 3-N-(morpholino) propane sulfonic acid |
| mRNA | Messenger ribonucleic acid |
| NTR | Nitroreductase |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| RIA | Radio immune analysis |
| RNA | Ribonucleic acid |
| s | Second(s) |
| SDS | Sodium dodecyl sulphate |
| SSC | Standard saline citrate |
| SV40 | Simian virus 40 |
| TAE | Tris/Acetate/EDTA |
| Tb | Body temperature |
| TE | Tris/EDTA |
| TEMED | N,N,N,N. tetramethylethylenediamine |
| UV | Ultraviolet |
| V | Volt(s) |
| WAT | White adipose tissue |

# 1 INTRODUCTION.

## 1.1 THE BIOLOGY OF WHITE AND BROWN ADIPOSE TISSUE.

Obesity refers to a complex and heterogeneous group of disorders in which there is an increase of the adipose tissue. This increase leads to a perturbation in the general metabolic state and as a result, obesity is a major risk factor for a number of clinical diseases, such as non-insulin-dependent diabetes mellitus (NIDDM), cardiovascular diseases, hyperlipidemia and hypertension. Because of these associated disorders, the life expectancy of obese people is greatly decreased. This has motivated new directions of research aimed at understanding the mechanism involved in the deposition and mobilisation of fat reserves. Many of these directions are underpinned by genetic and molecular biological techniques. The recent elucidation of the leptin pathway is a testament to the power of these new technologies.

Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue. The remaining cells are various blood cells, endothelial cells, adipose precursor cells of varying degree of differentiation and most likely fibroblasts (Geloen *et al.* 1989). These cells serve an important function in the energy economy of vertebrate organisms by providing a massive energy reserve that can be mobilised upon demand. Thus when caloric intake exceeds expenditure, metabolic flux is directed into pathways leading to triacylglycerol synthesis for storage in the adipocyte. Conversely when caloric expenditure exceeds intake, this energy reserve is mobilised to meet the deficiency and to provide needed physiological fuel (i.e. free fatty acids) for other cell types. Each of these specific lipogenic and lipolytic functions are realised by two types of adipose tissue known as white adipose tissue (WAT) and brown adipose tissue (BAT). In contrast to the development of BAT, which takes place mainly before birth, the development of WAT represents a continuous process throughout life and the acquisition of fat cells appears as an irreversible phenomenon. The capacity for white adipose tissue to store fat is a function of the repertoire of fat-specific genes which provides those

enzymes and proteins required for the transport and conversion of energy substrates into fat and others which enable it to be mobilised for the energy needs of the body (Kopecky *et al.* 1995). This function to store energy in its most concentrated form makes the white adipocyte one of the most energetically efficient cells in the body. Another adipocyte, the brown adipocyte, expresses a similar set of fat-specific genes plus a few others that enable it to produce heat as its major function.

The brown adipose tissue (BAT) has been proposed to play an important role in the regulation of energy balance (Himms-Hagen 1990). Unlike other tissues in which fuel oxidation is linked to the energy needs of the cell, BAT is distinguished by the unique presence of an uncoupling protein (UCP), a mitochondrial protein transporter that uncouples oxidative metabolism from ATP synthesis. This capacity permits BAT to expend calories unrelated to the performance of work, the net result being the generation of heat. Thermogenesis in BAT is under the control of the sympathetic nervous system via noradrenergic innervation of BAT cells (Himms-Hagen 1990). The neural signals emanate from the hypothalamus and are transmitted by the autonomic nervous system to regulate this highly innervated tissue, which employs the uncoupling protein (UCP), a 32 kDa protein from the inner mitochondrial membrane to uncouple mitochondrial respiration (Jezek *et al.* 1994). BAT activity is stimulated during cold exposure (non-shivering thermogenesis) and during the ingestion of excess calories (diet-induced thermogenesis). The role of BAT in mediating diet-induced thermogenesis led to the suggestion that BAT activity might provide protection from obesity. This was recently demonstrated, using genetic ablation techniques to generate mice with isolated brown fat deficiency (Lowell *et al.* 1993). Regulatory elements from UCP gene were used to direct the expression of the A subunit of diphtheria toxin to the brown fat. At the age of 16 days transgenic mice had markedly decreased functional brown fat mass, which was accompanied by moderate obesity, with a 31% increase in total body lipid. But as the transgenic mice aged, they showed excessive weight gain compared with control littermates and developed obesity in the absence of hyperphagia demonstrating the decreased energy expenditure of these mice.

## 1.2 ADIPOCYTE DEVELOPMENT AND TRANSCRIPTIONAL CONTROL *IN VITRO.*

The availability of immortalised cell lines (such as 10T1/2 stem cell line and the 3T3 and OB1771 preadipocyte lines), which represent different stages of adipocyte development, has greatly facilitated the characterisation of the differentiation program and the identification of many cis-regulatory elements and trans-acting factors involved in the coordinate expression of adipocyte genes during differentiation. Adipogenesis *in vitro* follows a highly ordered and characterised temporal sequence. Initially, there is growth arrest of proliferating preadipocytes, usually achieved in culture cell lines after contact inhibition. In culture cells models, initial growth arrest is induced by the addition of a prodifferentiative hormonal regimen, which includes insulin, glucocorticoids and an agent that increases the intracellular level of cAMP. Upon induction (day 0 in Figure 1.1), the cells undergo one or two additional rounds of cell division known as clonal expansion then become quiescent as the coordinate transcriptional activation of adipocyte genes is initiated and acquire early markers but do not yet accumulate triacylglycerol. C/EBPβ and C/EBPδ are expressed immediately following hormonal induction of differentiation. These two synergistically stimulate the expression of C/EBPα and peroxisome-proliferator-activated receptor γ (PPARγ) both of which contain C/EBP binding sites in their promoters. While C/EBPα can also induce expression of PPARγ, C/EBPβ is probably the initial inducer since it is expressed before C/EBPα in the differentiation program (see Figure 1.1). The induction of these two proteins is characterised by a second, permanent period of growth arrest followed by expression of fully differentiated phenotype. The exact mechanism by which PPARγ and C/EBPα bring about this change is unclear, but several important clues have appeared recently. In 3T3L1 cells, the shift from dividing preadipocytes to growth-arrested cells to fully differentiated fat cells was associated with changes in the expression of several cyclin-dependent kinase inhibitors, p18, p21 and p27 (Morrison and Farmer, 1999). The expression of PPARγ in NIH-3T3 fibroblast specifically induced p18 and p21. C/EPBα has also been shown to have antimitotic properties (Umek *et al.* 1991).

The process of terminal differentiation occurs over several days in cultured cell lines. This is followed by the appearance of PPARγ and C/EBPα, which activate de *novo* or enhance the expression of most or all of the genes that characterise the adipocyte phenotype. C/EBPα activates the expression of several adipocytes specific genes, a number of which have been found to have C/EBP binding sites in their promoter (Herrera *et al.* 1989). Some of these genes include glycerophosphate dehydrogenase, fatty acid synthase, acetyl CoA carboxylase, Glut 4, insulin receptor and aP2 (the adipocyte-selective fatty acid binding protein) (Spiegelman *et al.* 1993). PPARγ also transactivate the expression of some of these genes whose promoters contain regulatory elements for PPARγ. This process of terminal differentiation leads to the formation of triacylglycerol-filled, mature adipose cells that are accompanied by the emergence of α2-adrenergic-mediated antilipolytic response (Ailhaud *et al.* 1992). Throughout this process, lipid-laden droplets begin to appear in the cytoplasm, and over time they increase in size and often coalesce into one or few major droplets resembling the appearance of adipocytes *in vivo*.

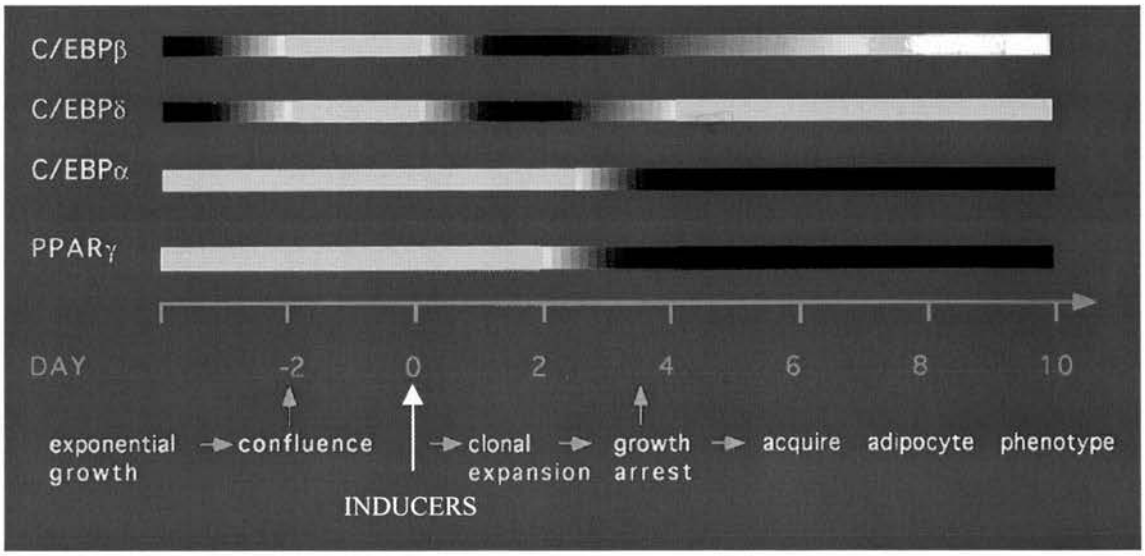



**Figure 1.1 Expression of C/EBP and PPAR family members during differentiation of 3T3L1 preadipocytes.** Magnitude of expression is indicated by the intensity of the horizontal band. (Modified from Mandrup and Lane, 1997).

## 1.3 HORMONAL REGULATION OF THE DIFFERENTIATION PROCESS.

After cell attachment in the presence of serum and the formation of an extracellular matrix, growth of adipoblasts and commitment to preadipose cells takes place in the presence of a few growth factors; insulin at high concentrations or IGF-I at low concentrations and basic fibroblast growth factor (bFGF) (Gaillard *et al.* 1984). Adipogenic hormones are only required subsequently for the terminal differentiation of preadipose to adipose cells, which corresponds to the formation of new fat cells. Among the circulating hormones, GH, acting as a protein kinase C (PKC) activator by means of diacylglicerol production from phosphatidylcholine, with activation of PKC pathway, is required for the terminal differentiation of 3T3-F442A and Ob17 cells, whereas in the presence of IGF-I, the adipogenic activity of GH can be mimicked by other PKC activators. Triiodothyronine (T3) also appears to be implicated in the terminal differentiation of Ob17 and 3T3L1 cells. Glucocorticoids, including the synthetic glucocorticoid dexamethasone, stimulate terminal differentiation of 3T3L1 and Ob17 preadipocytes as well as that of preadipocytes from rabbit and human (Hauner *et al.* 1989). Insulin modulates positively the differentiation program by affecting the expression of various proteins involved in triacylglycerol accumulation, such as GLUT4, fatty acid-synthesising and esterifying enzymes. Although a few differences can be observed between different species, the most common requirements for terminal differentiation appear to be IGF-I, insulin and glucocorticoids.

## 1.4 ADIPOSE TISSUE DEVELOPMENT *IN VIVO*.

During development the adipocyte acquires the full complement of enzymes and accessory proteins with which to carry out and regulate these lipogenic and lipolytic functions (Cornelius *et al.* 1994). The regulatory proteins include the receptors and second messenger system necessary to confer responsiveness to the hormones that regulate these processes e.g. insulin, which promotes lipogenesis, and counter-regulatory hormones such as glucagon, ACTH, epinephrine and glucocorticoids, which promote lipolysis. Although adipose tissue appears late in the embryonic development, the major expansion of the adipocyte population occurs

only after birth. This expansion coincides with the need for an energy reserve enabling the newborn to survive in a new environment in which the intervals between nutrient intake may be lengthy. The adipose lineage arises relatively late in the developmental pathway from a multipotent stem cell of mesodermal origin that also gives rise to the muscle and cartilage lineages. Previous studies on the development of white adipose tissue (WAT) and to a lesser extent of brown adipose tissue (BAT) have led to much controversy regarding the existence of distinct precursor cells and the interchangeability between both tissues. Most recent evidence supports the view that BAT and WAT are distinct organs containing distinct adipose precursor cells. At the cell level, the existence of distinct BAT precursor cells is demonstrated by their unique ability to express the uncoupling protein (UCP) gene (Kopecky *et al.* 1990). The anatomical location in various species indicates that BAT occurs at specific sites, for instance in interscapular and axillary regions, whereas WAT occurs also at specific but distinct sites e.g. subcutaneous, omental, perirenal and inguinal depots. The development of BAT and WAT in uterus and postnatally differs according to animal species. After birth, the growth of the various WAT depots has been shown in different animal species to respond differentially to age, diet and cold exposure. White adipose tissue cannot be detected macroscopically during embryonic life and at birth in rodents (rat, mouse), whereas it is present at birth in the pig, rabbit, guinea pig and humans. The development of white adipose tissue has been studied in pig fetuses (Hausman G.J. 1987). Approximately at the beginning of the last third of the gestation period, large and small arterioles can be detected in the vicinity of small developing fat cell clusters that are surrounded by extensive stroma. Cell cluster size increases steadily with fetal age, but the number of clusters does not change significantly before birth.

The embryonic development of human adipose tissue takes place earlier than that of the pig, i.e. at the beginning of the second third of the gestation period in various sites (buccal, neck, shoulder, gluteal, perirenal) (Burdi *et al.* 1985). Adipose tissue appears and develops in those areas where it remains after birth. Postnatal development of adipose tissue shows sex and site-related differences in body fat distribution in human and various animal species. The ability of adult rodents and human to increase the number of adipocytes depending on the localisation of the fat

depot, the nature of the diet, and the environmental conditions to which the animals were exposed, has been long known (Miller *et al.* 1983). Some controversy exists, however, as to whether the formation of new fat cells takes place during refeeding after a prolonged period of food deprivation. The hyperplastic development of adipose tissue in aging animals fed a high-carbohydrates or high-fat diet has thoroughly been studied. Miller *et al.* (1983) demonstrated the ability of rats to increase the fat cell number in most of their adipose depots (retroperitoneal, perirenal) in response to high-carbohydrates or high-fat diet. The life-long potential to make new fat cells has been clearly illustrated in rodents. For instance, the perirenal fat depots of very old mice from both sexes contain large amounts of early markers of differentiation, i.e. A2COL6/pOb24 mRNAs, LPL mRNAs and IGF-I mRNAs, indicating that preadipocytes at stages 2 and 3 are indeed present in these depots (Ailhaud *et al.* 1990). A similar conclusion has been found in humans, where a significant proportion of stromal-vascular cells from subcutaneous fat tissue of elderly men and women was found to differentiate *in vitro* into adipose tissue cells (Hauner *et al.* 1989). This finding indicates that adipose precursor cells, which are not likely to give rise to new fat cells, are indeed present in those individuals. This observation can also explain the acquisition of new fat cells, which is known to take place at the adult stage in normal subjects and obese patients.

## 2. GENERAL ASPECTS OF CELL ABLATION.

### 2.1 INTRODUCTION.

The mechanisms governing organogenesis and other morphogenetic processes in higher organisms involve complex interactions among different cell types. Both intrinsic and extrinsic factors may act to determine cell fate, rendering it difficult to discern the role that individual processes play in effecting cell commitment. The ability to ablate specific cell types during development provides an experimental approach for investigating these processes and assessing the specific contributions that cellular interactions play in determining cell fate. Although developmentally related, tissues may remain near one another in the adult

animal, it is also common for cells to relocate far from their initial origins. Thus, it is not possible to determine lineage relationships by examining adult tissues. A useful method for investigating the role of a specific cell type is to remove them and determine what effect this has on the normal organogenesis. This can be achieved by genetic ablation approaches, which uses transgenic technology to direct expression of intracellular toxins to certain cells during development (Breitman *et al.* 1987). This approach allows then to determine how the loss of these cells affects the development of the animal (Evans G. 1989, Palmiter *et al.* 1987, Camper *et al.* 1995).

The programmed ablation of specific cell types by genetic approaches provides a novel means of generating mutant animals that lack a specific cell type or an entire cell lineage. These animals thus provide a novel approach for asking questions about the role of specific cell types in physiological processes and cell fate during embryogenesis. As well, animals that lack specific cell types can be used to create models of certain human diseases.

The ablation of specific cell types in a transgenic animal relies on the fact that virtually all differentiated cell types in the body can be characterised by the expression of highly specialised proteins whose synthesis is restricted to that cell type. Through the use of gene transfer into various cell types in culture and more importantly into the mouse germ line, it has become clear that there exist cis-acting sequences located around the coding regions of genes that are responsible for ensuring that developmentally regulated genes are expressed in the appropriate cell types at the appropriate times during both embryological development and during cellular differentiation in the adult. The cis-acting sequences responsible for programming gene expression can be identified and dissected away from the structural regions of a gene and used to direct the expression of heterologous sequences, regardless of chromosomal location, in transgenic mice. Thus, cis-acting regulatory elements can be used to target expression of genes that encode toxic proteins to specific cell types in transgenic animals. Cancer chemotherapy provides a promising area where genetic ablation can be used. In this case, retroviral-mediated gene transfer could be used for the transduction of functional foreign genes into human cancer cells. If the inserted gene codes for drug sensitivity,

administration of an appropriate drug will selectively kill those cells expressing the gene.

## 2.2 STRATEGIES FOR GENETIC ABLATION.

Two main approaches have been generally used to achieve selective cell ablation. Physical methods, which involve physical destruction of target cells, as for example the UV-laser ablation (Lohs-Schardin *et al.* 1979), dye photo ablation (Miller and Selverson, 1979), current intracellular injection (Kuwada and Goodman, 1985), surgery (Smith TJ, 1989) and genetic ablation methods, which involve targeting expression of a potentially lethal toxin to a specific cell type in transgenic mice using appropriate gene promoter/enhancer.

With this last method, two main strategies have been devised to generate selective ablation of different cell types in transgenic mice. One involves the expression of toxin genes, such as the A subunit of diphtheria toxin or the A subunit of ricin. Expression of the transgene provides high levels of the toxin and results in cell death in all toxin-expressing cells. The second strategy involves the use of an enzyme-prodrug combination, which involves expressing a gene encoding an enzyme that converts an inactive prodrug to a cytotoxin. The most widely used gene in this approach is the thymidine kinase (*tk*) gene (Borreli *et al.* 1989) from herpes simplex virus, whose expression in animal cells and animals is only toxic in the presence of certain nucleoside analogous that can be metabolised only by the herpes enzyme.

More recently, a new system for achieving the inducible ablation of specific cell types in transgenic mice has been developed (Clark *et al.* 1997). The system is based on the *E.coli* nitroreductase enzyme (NTR), which activates certain nitro compounds such as the antitumor drug CB1954 to potent cytotoxic agents. The compounds generated are secondarily activated in cells by thioesters to very reactive species that cross-link DNA (Knox *et al.* 1993). DNA cross-linking results in cell death that is independent of cell division.

### 2.2.1 TOXINS.

**a) The A subunit of diphtheria toxin:**

Diphtheria toxin (DT) is synthesised by *Corynebacterium diphtheriae* (the causative agent in human diphtheria) as a precursor polypeptide that is secreted and enzymatically cleaved into two fragments, of 193 and 342 aminoacids (designed A and B chains), joined by a disulphide bridge (reviewed by Pappenheimer, 1977). The B subunit binds to the surface of most eukaryotic cells, where it is internalised by endocytosis and delivers the A chain (DT-A) into the cytoplasm. DT-A then catalyses the transfer of ADP-ribose from NAD to a modified histidine residue on elongation factor 2, thereby inhibiting protein synthesis and resulting in the cell death (Collier, 1975; Mohering *et al.* 1984; Kohno *et al.* 1986). Thus, the expression of the A subunit in eukaryotic cells, in the absence of the B subunit, results in cell death without killing neighbouring cells due to the release of the A subunit from dying cells. Once inside, the DT-A is extremely toxic: a single molecule has been shown to be enough to kill a cell (Yamaizumi *et al.* 1978).

The extreme toxicity of DT-A provides a powerful way of killing cells that express this protein. However, the exquisite sensitivity of eukaryotic cells to DT-A poses a potential problem, since its use for genetic ablation requires absolute lineage or cell type specificity of the promoter/enhancer used to drive expression in transgenic mice. Because of the extreme lethality, it is important to have very restricted expression of the targeted toxin. Low levels of expression in tissues required for fetal survival can lead to the death of the entire transgenic animal instead of producing selective ablation of the targeted cell type. Additionally, many genes are normally expressed at low level in a diverse set of tissues. Thus, death of the animal could result from an appropriate low level of transgenic expression. One way to avoid this has been to use an attenuated form of the diphtheria toxin, tox 176 (see below).

**b) Attenuated diphtheria toxin (tox 176):**

Tox 176 has a single aminoacid substitution (G/D) which makes it ~30 fold less active (Breitman *et al.* 1990). This attenuated toxin has been successfully used in transgenic toxin studies (Lowel *et al.* 1990; Breitman *et al.* 1990 and Ross *et al.*

1993), but limited comparative information has been available, with the only exception perhaps being the work of Burrows *et al.* (1996). They prepared constructs using 4.6 kb of the α-GSU promoter to direct expression of both forms of the diphtheria toxin to pituitary gonadotrope and lactotrope cells and compared viability and ablation efficacy between the two sets of transgenic mice. These authors found that the attenuated version of the diphtheria toxin gene is a better choice for transgenic toxin studies because of its decreased lethality and equivalent ablation. In contrast, Lowell *et al.* (1993) observed regression of the phenotype in mice after ablation of the brown fat tissue with this attenuated toxin, an effect that was not observed with the diphtheria toxin A subunit. Thus, what has been proved successful in some cases may not necessary work in others.

**c) Ricin:**

Ricin is produced by the castor bean *Ricinus communis* as a nontoxic precursor preproricin that is processed and activated by protease activity (Sandvig and Olsnes, 1982). Mature ricin consists of a 262 aminoacid B polypeptide, containing galactose-specific cell-surface binding and membrane translocation activity, disulphide cross-linked to a 267-aminoacid toxic A subunit, which is a N-glycosidase specific for A4324 of ribosomal 28S RNA (Endo *et al.* 1987). Internalisation of the A subunit, mediated by cell-surface binding of the B subunit, catalytically destroys ribosome function and blocks protein synthesis. In the absence of the binding and internalisation functions inherent in the B subunit, the ricin A subunit remains in the extracellular space where its toxicity is extremely low (Pastan *et al.* 1986). This property is important for the precision of genetic ablation in transgenic mice, because the release of endogenously synthesised A chain during genetically programmed cell death will not kill surrounding cells.

### 2.2.2 ENZYME PRODRUGS COMBINATIONS.

**a) Thymidine kinase (*tk*)-Gancyclovir system:**

In this strategy it is possible to control the timing of ablation by administering the prodrug at appropriate times. Expression of HSV-*tk* is not by itself, harmful to cells, but it is capable of selectively catalysing the incorporation of certain

nucleoside analogues, such as 1-(2-deoxy-2fluro-β-D-arabinofuranosil)-5-iodouracil, gancyclovir and acyclovir, into host cell DNA resulting in cell death. Limitations of this approach are ectopic expression in the testis, which results in difficulties in the establishment of breeding lines (Al-Shawi *et al.* 1991) although a new truncated version of the herpes simplex thymidine kinase gene has recently demonstrated to allow the generation of fertile homozygous mice (Cohen *et al.* 1998). Another drawback is the belief that HSV-*tk* kills only proliferating cells but not differentiated cells. However, this view has also been challenged by a number of workers who have described effective inducible ablation in tissues with low mitotic indices (Wallace *et al.* 1991; Allen *et al.* 1995). In these cases the mode of action of the activated prodrug is not clear, although in non-proliferating thyrocytes it apparently occurs by p53-independent apoptosis (Wallace *et al.* 1996).

**b) Nitroreductase (NTR)-CB1954 system:**

One of the main drawbacks of the inducible system described earlier is the large dose of active drug that needs to be present for long periods in order to produce cytotoxicity. In addition to this, non-cycling cells are resistant to these agents. Alkylating agents, on the other hand, do not suffer from these intrinsic drawbacks. A suitable prodrug-enzyme combination using one of these alkylating agents (NTR-CB1954 system) has demonstrated that can generate highly cytotoxic species, which are not cell cycle specific. Furthermore these species may have a bystander effect so that they migrate and kill surrounding cells, non-infected tumour cells, in the case of gene therapy or non-expressing cells when used to ablate specific normal cells, for example in a transgenic animal model (Knox *et al.* 1988; Clark *et al.* 1997; Drabek *et al.* 1997).

Whilst chemically only a monofunctional alkylating agent (by virtue of its single aziridine function), CB1954 exhibited a dramatic and highly selective activity against the rat Walker 256 tumour (a rat mammary gland tumour cell line) and could actually cure this tumour. The hope that a human tumour could be found that shared the Walker tumour's sensitivity has made the mechanism of action of CB1954 the focus of continual research for over 30 years (for a review see Knox *et al.* 1993a).

Although CB1954 showed high sensitivity to Walker tumour cells both *in vitro* and *in vivo,* it was soon established that CB1954 was ineffective in a range of animals and human tumours (Workman *et al.* 1986). Roberts *et al.* (1986) shown that the basis of this sensitivity is that CB1954 can form DNA interstrand cross-links in Walker cells but not in insensitive cells. All steps in the bioactivation of CB1954 have been elucidated and the intermediates synthesised and studied for biological activity. The initial step is the reduction of CB1954 (Figure 1.2) by the rat form of the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone), EC 1.6.99.2 to form 5-aziridin-1-yl-4-hydroxylamino-2-nitrobenzamide (Knox *et al.* 1988). This is the active derivative of CB1954, which can then react with thioesters in the cytosol of the cells to produce DNA reactive species, that form DNA interstrand cross-links (Knox *et al.* 1991). The major product of this reaction is however 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide that does not react readily with DNA and therefore is in competition with the production of DNA binding products (Knox *et el.*, 1991).

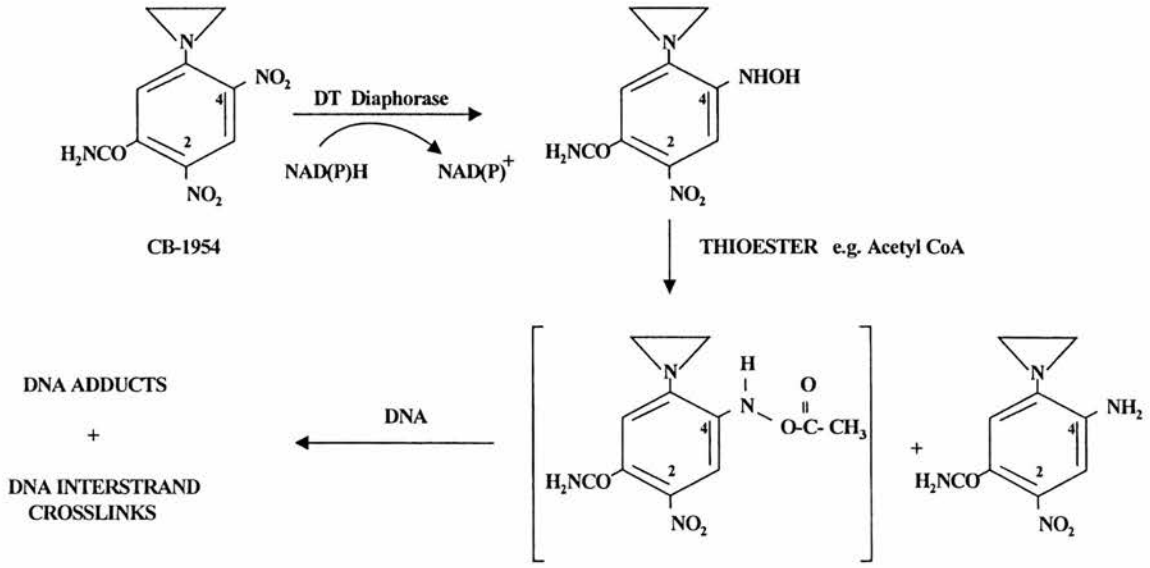



**Figure 1.2.** Bioactivation of CB1954 by the DT-diaphorase.

These DNA interstrand cross-links are formed with very high frequency and can contribute up to 70% of the total lesions (Friedlos *et al.* 1992). These interstrand cross-links formations are generally accepted as the most toxic lesion, being poorly excised by the cell and therefore inhibiting DNA function resulting in cell death. The marked increased cytotoxicity of CB1954 has been suggested to be due to some unusual properties of these interstrand cross-links. In fact, molecular modelling studies carried out by Friedlos *et al.* (1992) showed that the 4-hydroxylamine, (after activation as detailed above) reacts predominantly with the C8 position of deoxyguanosine, thus leaving the aziridine function in the DNA available to react preferentially with the O6 position of a deoxyguanosine on the opposite strand of DNA.

A number of human cell lines were shown to contain DT diaphorase levels comparable to those found in Walker and some other rat cell lines (Boland *et al.* 1991). The rat cell lines were all sensitive to CB1954 and the resulting cell killing approached that obtained in Walker cells. Human cell lines however, were much less sensitive to CB1954 with 500 to 5000 higher dose of the agent being required to produce a comparable cytotoxic response. In contrast, both the rat and human cell lines were similarly affected by the 4-hydoxylamino derivative of CB1954 (Boland *et al.* 1991). This fact showed that the resistance to CB1954 was not due to any failure to activate further the hydroxylamine or to an intrinsic resistance to the DNA adducts formed. It was therefore suggested that CB1954 was reduced differentially by the human form of DT diaphorase as compared to the rat form (Boland *et al.* 1991). The human form of DT diaphorase was purified to homogeneity from Hep G2 cells and significant differences could be observed in its ability to reduce CB1954 to the active 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide derivative as compared to the rat form. Although, both forms of the enzyme produced the 4-hydroxylamino derivative as the single product, the human Hep G2 form of the enzyme was intrinsically less able to carry out this reduction and the $K_{cat}$ value was over six-fold higher for the Walker cell form of the enzyme (4.1 $min^{-1}$) than for the human DT diaphorase (0.64 $min^{-1}$) (Boland *et al.* 1991). The intrinsic inability of human DT diaphorase to produce the required cytotoxic species from CB1954 accounts for the lack of sensitivity of human cells towards this agent. To

overcome this intrinsic resistance of human cells to the prodrug CB1954, it has been proposed that the activating enzyme could be targeted towards a human tumour by conjugating it to a localising antibody, such a strategy is known as antibody directed enzyme prodrug therapy or ADEPT therapy (Knox *et al.* 1993). For this approach a nitroreductase enzyme has been purified and cloned from *E.coli* (Anlezark *et al.* 1992).

NTR like DT diaphorase is also capable of reducing CB1954 in air to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (Knox *et al.* 1992). In contrast to DT diaphorase, which can only reduce the 4-nitro group of CB1954, NTR can reduce either (but not both) nitro groups of CB1954 to the corresponding hydroxylamino species, these are formed in equal proportions and at the same rate. However, no products are formed in which both nitro groups have been reduced. Thus, once one nitro group has been reduced, NTR can not then reduce the other nitro group (Knox *et al.* 1992). 5-(Aziridin-1-yl)-4-hydroxylamino-2–nitrobenzamide is a potent cytotoxic drug capable of producing DNA-DNA interstrand cross-links in cells (Knox *et al.* 1988). In contrast the 2-hydroxylamino species is less cytotoxic and can not produce interstrand cross-links. It is however, much more cytotoxic than CB1954 itself (Knox *et al.* 1988). CB1954 is reduced about 90-fold more rapidly by NTR than by DT diaphorase (Figure 1.3).

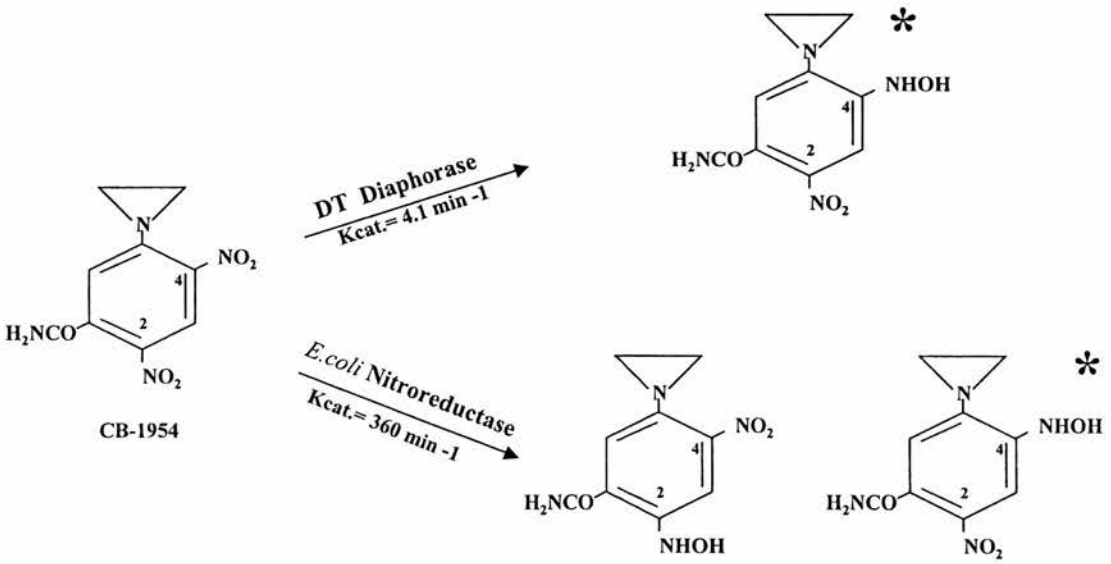



**Figure 1.3** Bioactivation of CB1954 by the *E.coli* nitroreductase.

As well as being able to reduce CB1954, NTR shares some other biochemical properties with DT diaphorase. It can reduce menadione and utilises either NADH or NADPH as a cofactor. However, it is a much smaller protein (24kD) than DT diaphorase (33.5kD) and there is no obvious sequence homology between the two enzymes (Anlezark *et al.* 1992).

Recently, new possibilities for the use of CB1954 have arisen with the advent of antibody-directed enzyme prodrug therapy or ADEPT (Knox *et al.* 1993) and gene-directed enzyme prodrug therapy or GDEPT (Connors TA 1995). In these approaches either *E.coli* nitroreductase or the gene encoding this enzyme are delivered to the tumour site where they locally activates a prodrug. Bridgewater *et al.* (1995) have used a recombinant retrovirus encoding the *E.coli* NTR gene to infect mammalian cells. Murine (NIH3T3) cells expressing NTR were killed by the prodrug CB1954, demonstrating that the system is effective in non-cycling cells. In the same report NTR expression in human melanoma, ovarian carcinoma or mesothelomia cells also rendered them sensitive to CB1954 killing. A number of studies attempting gene delivery to tumours *in vivo* have shown that a proportion of cells remain unmodified (Ram *et al.* 1993). Gene delivery is probably limited both by the efficiency of various current gene delivery technologies and by restricted access to some tumour regions. Thus, a desirable feature of enzyme/prodrug combinations for use in GDEPT is that the killing of modified cells result in the killing adjacent unmodified cells. This "bystander" effect described with TK/GCV system (Freeman *et al.* 1993) was also observed by Bridgewater *et al.* (1995).

Improved cytotoxicity and bystander killing was achieved by simultaneous use of both NTR/CB1954 and TK/GCV systems. Two other articles showed the potential of the NTR/CB1954 system not just in cells in culture but also *in vivo* (Clark *et al.* 1997; Drabek *et al.* 1997). Both groups directed expression of the bacterial enzyme to specific cellular targets by placing the gene under the transcriptional control of tissue-specific elements. In the work of Clark *et al.* (1997) the ovine β-lactoglobulin promoter directed expression of the luminal cells of the mammary gland whereas in Drabek *et al.* (1997)'s studies control elements of the human CD2 locus restricted nitroreductase expression to T cells. This targeted destruction of particular cell types allowing the ablation of selected tissues offers a

powerful tool for many types of studies. Though, as both groups point out, it is the possibility of using the nitroreductase (NTR) gene in anticancer therapy, which appears to offer greatest promise.

The cytotoxic effects of the prodrug in a mouse model *in vivo* were observed relatively shortly after prodrug administration, i.e. by 48 hr (Clark *et al.* 1997). Following drug administration *in vitro* Drabek *et al.* (1997) observed morphological changes in murine L cells, that included the generation of enlarged mulinucleate cells. The authors proposed that the changes could be due to a block in DNA synthesis with continued RNA and protein synthesis. DNA strand breaks and interstrand cross-links were observed in CB1954-treated cells (Clark *et al.* 1997) suggesting that these may be involved in the mechanism of cytotoxicity. As a consequence of these lesions in the genome of the cells a possible mechanism of apoptosis has been suggested to be involved. Thus, Drabeck *et al.* (1997) who targeted NTR expression to T cells using the CD locus control region (LCR), reported an increased proportion of apoptotic thymocytes in CB1954 treated mice relative to controls. The same group suggested that the killing of non-expressing B cells was a consequence of an *in vivo* bystander response while in Clark's studies mammary luminal cells were ablated leaving closely associated myoepithelial cells unaffected. Clearly the underlying fact is what may be a desirable attribute under one set of circumstances need not be so desirable under other circumstances. Thus in cell-lineage studies the aim would be to minimise the bystander effect whereas in tumour destruction the aim would be to maximise the response. These two studies clearly demonstrated the feasibility to of the NTR/CB1954 system to kill specific cells in an animal model.

## 3 *IN VIVO* ABLATION.

### 3.1 INTRODUCTION.

A number of systems have been described where complete cell lineages have been ablated in transgenic mice, that carry constructs in which a cellular toxin is linked to a tissue-specific promoter (as it as been described above). Such experiments with the diphtheria toxin A and elastase I promoter have succeeded in ablating the exocrine pancreas (Palmiter *et al.* 1987), and with the toxin and the

promoter for crystalline to have resulted in destruction of lens tissue (Breitman *et al.* 1987). The reader is referred to Berstein and Breitman, (1989) for a comprehensive review, as this chapter will only describe the ablation of the pituitary gland as an example where by using some of the systems described above it has been possible to develop models for analysis of very specific interactions between cells of the pituitary gland. More detail will be given on the description of the studies of ablation and manipulation of the adipose tissue stores, as it is the most relevant example to this project.

## 3.2 PITUITARY ABLATION.

The anterior pituitary gland (or adenohypohysis) develops from an ectodermal bend that protrudes from the roof of the oral cavity and becomes juxtaposed to a neuroectodermal extension from the base of the brain. The posterior pituitary synthesises and secretes polypeptide hormones such as growth hormone (GH), prolactin (PRL), gonadotropins (FSH and LH), corticotropin (ACTH) and thyrotropin (TSH) (Simmons *et al.* 1990). The hormones of the mature pituitary are expressed in a specific temporal pattern during the organogenesis, with the entire complement present at birth. The ordered appearance of these cell types has made the anterior pituitary an interesting model for studying cell lineage relationships. One approach to examining the origins of various cell types in the pituitary has been the generation of transgenic mice in which the expression of Diphtheria toxin A chain (DT-A) gene has been targeted by the growth hormone (GH) promoter/enhancer sequences, (Behringer *et al.* 1988). In this study a line of transgenic mice was established which lacked detectable levels of circulating GH. This deficiency resulted in dwarfism, and a very marked reduction in the number of GH-positive somatotrope cells in the pituitary. There was also a large decrease in the number of lactotropes in these mice, suggesting that both somatotropes and lactotropes derive from a common precursor cell. Later, Borrelli *et al.* (1989) generated transgenic mice carrying the herpes *tk* gene under the control of either the rat growth hormone (GH-*tk*) or the rat prolactin (PL-*tk*) promoter. Treatment of the GH-*tk* mice FIAU resulted in dwarfism and ablation of both somatotropes and

lactotropes. In contrast, transgenic mice expressing HSV1-*tk* in the lactotropes treated with FIAU had anatomically and histologically normal pituitaries. On the basis of these observations, the author concluded that somatotropes and lactotropes derived from a common cycling stem cell pool that is already committed to GH, but not prolactin expression. This was thought to be due to the fact that prolactin expression is activated postmitotically in the lactotropes so making them refractory to cell killing.

## 3.3 ADIPOCYTE ABLATION AND TRANSGENIC AND KNOCKOUT RODENT MODELS OF ALTERED BODY FAT DISTRIBUTION.

### 3.3.1 INTRODUCTION.

The ability to genetically alter the expression of adipocyte genes and control the fatness of feed animals remains as an important goal of agricultural research. Adipocytes play a central role in maintaining the energy balance of vertebrate animals. These cells store energy in the form of triglycerides during periods of nutritional abundance and release it in a biochemical useful form at times of nutritional deprivation. Overall excess of body fat as well as regional adiposity are recognised risk factors for type 2 diabetes, dyslipidemia, hypertension, and coronary heart disease. Other disorders with extreme abnormalities in body fat distribution, such as familial and acquired lipodystrophies, are also frequently associated with marked insulin resistance, early-onset diabetes mellitus, hypertriglyceridemia, and low serum concentration of high density lipoprotein (HDL) cholesterol (Foster, D. 1998).

Earlier attempts to reduce adiposity have included the use of hormones such as growth hormone and β-adrenergic agonists due to their lipolytic and thermogenic properties. Both schemes have been shown to improve carcass composition (Futter and Flint 1987), however they are not free of unwanted side effects (Warriss *et al.* 1990) and increased consumer resistance has ended with these approaches being banned, for agricultural purposes, in Europe and other countries. This situation directed the attention of some researches to develop new technologies to overcome

some of the problems associated with the use of hormones. One of such approaches includes the use of antibodies to target adipocytes for destruction with the immune system (Flint *et al.* 1986). These early studies were conducted in rats using antibodies to adipocytes plasma membranes raised in sheep. Injection of antibodies produced a significant decrease in adiposity mass for up to 8 weeks (Futter and Flint, 1987). Furthermore, beneficial effects, in agricultural context, could also be observed in terms of increased appetite, protein deposition and feed conversion (Panton *et al.* 1990), which has extended the focus of attention of this technology to other species including sheep, pigs, chickens, and rabbits (for review see Flint DJ, 1996).

Recently, great progress has been made in identifying several genes and understanding the molecular mechanisms underlying spontaneous syndromes of obesity and abnormal body fat distribution in animal models and humans. Progress has also been made in establishing transgenic animal models with altered body adiposity and peculiar body fat distribution similar to that seen in familial forms of human lipodystrophy. Table 1.1 displays several mouse models with either transgenic or knockout of specific genes that alter the amount or the distribution of body fat. These animal models not only reveal that certain genes may affect body adiposity by influencing energy balance, but also indicate the critical role of genes in adipocyte proliferation and differentiation.

**Table 1.1** Transgenic and knockout models of obesity and altered body fat distribution.

| Models with Increased Body Fat or Obesity. | |
|---|---|
| Overexpression of *glut*-4 gene in adipose tissue. | (Shepherd *et al.* 1993) |
| Overexpression of AGRP gene. | (Graham *et al.* 1997) |
| Ablation of brown fat. | (Lowell *et al.* 1993) |
| Inactivation of GH transgene. | (Pomp *et al.* 1996) |
| Knockout of neuropeptide Y-Y1 receptor gene. | (Kushi *et al.* 1998) |
| Knockout of β3-adrenergic receptor gene. | (Susulic *et al.* 1995) |
| Knockout of Mc4-r gene. | (Huszar *et al.* 1997) |
| Knockout of ICAM-1 gene or integrin α-Mβ2 gene. | (Dong *et al.* 1997) |
| Knockout of bombesin receptor subtype-3 gene. | (Hamazaki *et al.* 1997) |
| Knockout of PPARα gene. | (Costet *et al.* 1998) |
| Knockout of 5-$HT_2$ serotonin receptor gene. | (Tecott *et al.* 1998) |

Models with Decreased Body Fat or Lipodystrophy.

| | |
|---|---|
| Antibodies to adipocytes membranes. | (Flint *et al.* 1986) |
| Overexpression of LPL gene in muscle and cardiac tissues. | (Levak-Frank *et al.* 1995) |
| Overexpression of β1- adrenergic receptor gene. | (Soloveva *et al.* 1997) |
| Overexpression of uncoupling protein gene. | (Kopecky *et al.* 1995) |
| Overexpression of A-Zip/F gene. | (Moitra *et al.* 1998) |
| Overexpression of nSREBP-1c gene. | (Shimomura *et al.* 1998) |
| Knockout of the *glut*-4 gene. | (Katz *et al.* 1995) |
| Knockout of C/EBPα, β, δ genes. | (Wang *et al.* 1995 and Tanaka *et al.* 1997) |
| Ablation of thyroid hormone production. | (Bunger *et al.* 1998) |
| Knockout of RII β subunit of protein kinase Λ gene. | (Cummings *et al.* 1996) |
| Knockout of *mg* gene. | (Dinulescu *et al.* 1998) |

(Adapted from Chen and Garg 1999)

Studies of the differentiation of adipocytes *in vitro* have shown that this process is accompanied by the transcriptional activation of many genes, some of which have been cloned at cDNA level (Spiegelman *et al.* 1983; Chapman *et al.* 1984). As expected, many of these encode gene products that are involved in lipid metabolism. One of these gene products is adipocyte P2 (aP2), a very abundant adipocyte-specific member of the large family of intracellular lipid carrier proteins that includes the liver, heart and intestinal fatty acid binding proteins (Cook *et al.* 1988). The gene for adipocyte P2 (aP2) has been used as a model for understanding gene regulation in this cell type. Ross *et al.* (1990) found the first cis-acting regulatory sequences that can specifically direct gene expression to adipocytes *in vivo*. In that report, they found that the specific expression of the gene for aP2 in adipocytes derives from an enhancer element at kilobase (kb) -5.4 that directs linked chloramphenicol acetyltransferase (CAT) marker gene expression very strongly and specifically to fat tissue. Further analysis of this fragment defined a smallest fragment (-4.9 to -5.4) to the distal end of the 5.4 kb fragment as the primary determinant of the tissue-specific expression of this gene. This 500 bp enhancer directed expression of the CAT gene in a differentiation-dependent fashion when

linked to its own minimal promoter and stimulated very strong and highly specific expression from CAT gene in the adipose tissue of transgenic mice. Since its identification, this enhancer has been used in transgenic animals to direct adipose-specific expression of a number of genes that alter the biological function of this tissue including, SV40 large T antigen (Ross *et al.* 1992), diphtheria toxin A chain (Ross *et al.* 1993), and the insulin sensitive glucose transporter GLUT4 (Shepherd *et al.* 1993).

Multiple cis-acting elements within the 518 bp enhancer have been shown to be important for its differentiation-dependent activity in cultured 3T3-F442A preadipocytes and adipocytes. Initial studies identified a binding site (ARE1) for a member of the NF-1 transcription factor family. This NF-1 site appears to contribute to the overall activity of the enhancer, but is not functional as an isolated element, and the enhancer retains differentiation-dependent activity in the absence of this site (Graves *et al.* 1991). Further analysis established the presence of 4 additional cis-acting elements, each of which is also required for full enhancer activity in transient transfection assay (Graves *et al.* 1992). One pair of elements, ARE2 and ARE4, binds a ubiquitous positive-acting factor termed ARF2. A second pair of elements, ARE6 and ARE7, binds a separate nuclear factor termed ARF6 that is detected in nuclear extracts derived from adipocytes. Cloning of this factor identified it as a heterodimer consisting of PPARγ (Peroxisome proliferator-activated receptor) and its obligate partner, RXR (retinoid X receptor) (Tontonoz *et al.* 1994). These findings constituted the demonstration of the first adipocyte specific transcription factor. Thus, the demonstration that this DNA element directs strong and specific expression to adipose cells *in vivo* has opened up the possibility of experiments to genetically manipulate the adipose tissue *in vivo.* Some of these experiments include the delivery of toxic genes to the adipose tissue (Ross *et al.* 1993, Burant *et al.* 1997), expression of transgenes that can alter the amount or distribution of body fat by influencing energy balance, or disrupting adipocyte differentiation or proliferation (Soloveva *et al.* 1997, Moitra *et al.* 1998, Shimomura *et al.* 1998) and finally the delivery of transgenes that are non-toxic themselves but that can be activated by the downstream administration of a substance (prodrug) that make them

capable of selective cell killing once they are activated as it is the NTR-CB1954 system (Clark *et al.* 1997) that was used in this research project.

### 3.3.2 MICE MODELS WITH REDUCED ADIPOSE TISSUE STORES.

Ross *et al*, (1993) developed a mouse model with reduced adiposity to investigate the interrelationships between excessive adipose tissue mass and the disorders associated (diabetes, hypertension and hyperlipidemia). Reduction of the adipose tissue was achieved by the targeted expression of an attenuated diphtheria toxin A chain (described above) to adipose tissue, using the 5' regulatory region of the adipocyte P2 (aP2) gene. Transgenic mice with high levels of toxin expression developed chylous ascites and died shortly after birth. Transgenic mice expressing lower levels of the transgene had normal adiposity and survived to adulthood, however, these mice showed a loss of adipose tissue while they aged, which was accompanied by a progressive increase in serum glucose and insulin levels that culminated in the development of frank diabetes at approximately 8 months of age. Other characteristics of these mice were a complete resistance to chemically induced obesity. Nevertheless, these animals developed hyperlipidemia equal or greater than their non-transgenic obese littermates. Despite these diabetic symptoms, the authors could not pin down the link between the lack of fat and diabetes because these mice did not lose fat cells until they reached middle age.

Two recent experiments do a better job in reducing fat from animals. Both Shimomura *et al.* (1998) and Moitra *et al.* (1998) genetically blocked the growth of fat cells by altering transcriptions factors, proteins that turn genes on or off and are crucial to cell growth and differentiation. Moitra *et al.* inactivated the genes of two families of transcription factors that normally help fat cells grow and develop. They used tissue-specific expression of a dominant-negative protein transgene termed A-ZIP/F, that inhibits the DNA binding and function of B-ZIP protein in both the C/EBP and AP-1 families of transcription factors. Shimomura *et al.* on the other hand, altered the gene for another transcription factor so that the cells would overexpress it. Both mutations were designed to affect only fat cells, as the regulatory elements of the aP2 gene were again used in these studies to get the

specific expression in the fat cells. The changes produced by these two teams have similar effects: The mice were born with little or no white adipose tissue. The transgenic mice also failed to develop mature brown fat, which normally serves as a warming function in hibernating animals. Many of the animals died before they reached adulthood, but those that survived developed diabetes. Their cells no longer responded properly to insulin, as a result insulin levels in the bloodstream were increased up to 442 times normal levels for some mice and glucose levels at least tripled. Like human diabetics, the animals also had high levels of triglycerides and other fat building blocks in their circulation, and their organs became enlarged with the liver showing the presence of triglycerides.

### 3.3.3 MICE MONOGENIC MODELS OF OBESITY.

Body weight and body composition have been extensively studied in mice as model mammalian quantitative traits (Falconer, 1992). However, the number, location, and effects of individual genes contributing to variation in those traits are mostly unknown. A number of well described spontaneous monogenic rodent models of obesity syndromes have been known for decades, but only in the last 7 years have the molecular basis and the underlying pathophysiological mechanisms of obesity in these animals been elucidated.

#### a) The *agouti* gene mutation.

*Agouti* was the first of the obesity genes to be cloned (Bultman *et al.* 1992) and encodes a novel 131 residue secreted protein a, agouti signalling protein (ASP). The mRNA has been detected in many tissues although it is only known to function in the skin of wild-type mice in the neonatal period (Yen *et al.* 1994). It induces pheomelanin production in melanocytes to form the characteristic banded pattern of coat hair (yellow subapical band in black and brown hair). Dominant mutations, such as $A^{y}$ and $A^{vy}$, in the promoter region of the *agouti* gene result in its ectopic overexpression in all tissues (Bultman *et al.* 1992). Mice with this mutation exhibit

yellow coat colour, hyperphagia, obesity, increased lean body mass, hyperinsulinemia and infertility (Yen *et al.* 1994).

**b) The *tubby* gene mutation (*tub*).**

This phenotype is due to a point mutation that results in an error in the transcript splicing and the loss of the carboxyl terminus of the protein product (Kleyn *et al.* 1996). The mice homozygous for *tub* mutation develop late-onset obesity without diabetes (Coleman and Eicher, 1990). Affected mice also develop slow-onset deafness and retinal degeneration. This is in contrast to obese and diabetes mice that begin rapid weight gain soon after birth. Although *tubby* obese phenotype is inherited in a recessive manner, some significant differences have been observed in plasma triglycerides and susceptibility to arteriosclerosis in heterozygous mice.

**c) The carboxypeptidase E (Cpe) gene mutation (*fat*).**

The mutation underlying the fat phenotype has been shown to be within the gene encoding carboxypeptidase E (CPE), a prehormone-processing enzyme. The T729C nucleotide point mutation in *Cpe* gene causes a Ser202Pro substitution in CPE and renders this protein enzymatically inactive. CPE is present in many tissues, including the brain, pituitary, pancreas, and adrenals and is involved in the post-translational processing of many pro-hormones and neuropeptides, including proinsulin and POMC (Cool *et al.* 1997, Cool *et al.* 1998). Mice with homozygous *fat* mutation develop late-onset obesity, infertility, and hyperproinsulinemia (Coleman and Eicher, 1990).

**d) The leptin (*Lep*) gene mutation (ob).**

Recessive mutations in the mouse *obese* gene results in early-onset obesity in a syndrome resembling morbid human obesity (Coleman and Burkart, 1977). In addition, affected mice exhibit hyperphagia, hypothermia, hypercorticosteronemia,

decreased linear growth, and infertility (Coleman and Burkart, 1978). Cross-circulation experiments (parabiosis), over 20 years ago, suggested that the *obese* gene was likely to encode a circulating factor acting on a receptor (encoded by the *diabetes* gene) to regulate food intake (Pelleymounter M, 1995). The obese gene was eventually cloned in 1994 and the protein product named leptin (Zhang *et al.* 1994).

The *Lep* gene encodes a 167 amino acids (16 kD) protein, member of a class I of cytokine superfamily. The *ob* mutation is a single base substitution (C428T) that results in premature termination of the protein synthesis at codon 105 (Zhang *et al.* 1994). Although *ob* trait is autosomal recessive, mice heterozygous for *lep* mutation (*ob*/+) are more obese and hypoleptinemic than the wild-type (+/+) littermates (Chung *et al.* 1998).

Leptin is secreted by both white and brown adipose tissue cells in proportion to fat mass (Flier J.S, 1997). Leptin administration normalises all aspects of obesity and diabetes syndrome and restore reproductive function in *ob*/*ob* mice by acting through leptin receptors in the central nervous system (Campfield *et al.* 1995, Pelleymounter *et al.* 1995).

**c) The leptin receptor (*Lepr*) gene mutation (db).**

Mice carrying mutations at the *diabetes* locus exhibit a very similar phenotype to *obese* mice but they are resistant to leptin (Coleman, 1978; Pelleymounter *et al.* 1995, Halaas *et al.* 1995). The mutation resides in leptin receptor, a member of class-1 cytokine receptor family. Leptin receptor exists in several isoforms due to alternative splicing of the transcript. Ob-Rb, the long form expressed most highly in the hypothalamus, is the main isoform mediating leptin signalling. It consists of a large extracellular domain, a single transmembrane domain, and an intracellular domain that contains a motif important for interaction with Janus kinase (JAK) and a motif of signal transducer and activator of transcription (STAT). The mouse and the human leptin receptor (OB-R) molecules share 71% identity in intracellular domain. The *db* mutation is a single base substitution (G-T) in the intracellular domain of ob-Ra, which creates a splice site homologous to that in the intracellular domain of ob-

Rb. This results in the insertion of the C-terminus of ob-Ra into the cytoplasmic tail of ob-Rb transcript and subsequent premature termination of the ob-Rb synthesis (Lee *et al.* 1996).

## 4 AIMS AND APPROACHES OF THE PROJECT.

The general aim of this project was to assess the efficacy of CB1954 mediated ablation in adipocytes by directing the expression of the *E.coli ntr* gene to the adipose tissue using an adipocyte specific promoter. It was envisaged that an inducible model for the adipocyte ablation would provide a novel system for the study of fat metabolism and it would allow to investigate the interrelationship between excessive adipose tissue mass and the disorders associated (diabetes, hypertension, hyperlipidemia) through manipulation of the target cell population.

It was anticipated that such system would overcome some of the problems normally encountered with other suicide systems and provide a more flexible model where to study the physiology of adipose tissue at different stages of the mouse development including *in utero*, juvenile and adult. The system to be described is cell-type specific, it is effective in non-dividing cells and cell killing is inducible therefore this system affords the practical advantage of allowing stable pedigrees to be established before the initiation of the ablation studies. It also provides the means to control the timing and duration of toxic insult and to assess the potential for recovery of an ablated cell population after a prodrug free period in the transgenic animal.

This thesis also describes the preliminary experiments carried out in 3T3L1 cells to assess a novel approach of cell-mediated ablation, which was envisaged it could provide an alternative system for the study of the adipose tissue physiology and development.

# 2. MATERIALS AND METHODS

## 2.1 DNA MANIPULATION.

### 2.1.1 RESTRICTION DIGESTION OF PLASMID DNA.

Digestions of DNA with restriction endonucleases were carried out according to the conditions recommended by the manufacturers. Restriction enzymes were supplied by Boehringer Mannheim, NEB and Gibco BRL. Normally, 1 μg of DNA was digested in 10-20 μl volumes, using 1-2 units of enzyme for 90 minutes. When two different enzymes were used, both of which required the same buffer, the digests were carried out simultaneously. Otherwise, the lower salt concentration digest was carried out first and then the salt concentration increased for the second digest. When necessary, the reactions were terminated by heating to 68°C or 80°C for 15 minutes, according to the heat sensitivity of the enzyme. Restriction digests, which were to be run on gels, were terminated by addition of 0.1 volume of "stop mixture" (100 mM EDTA, pH 8.0, 20 % Ficoll and 0.1 % orange G).

### 2.1.2 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GELS.

DNA fragments were purified using the QIAquick gel extraction kit (QIAGEN) according to the instruction of the manufacturer. Briefly, after running the DNA sample, the gels were viewed on a middle range UV transiluminator and the required fragments excised in the smallest possible amount of agarose, using a sterile scalpel blade. The exposure to UV light was kept at a minimum in order to prevent excessive nicking of the DNA. The excised agarose containing the DNA fragment was weighed and 3 volumes of Buffer QG was added to 1 volume of gel (100 mg~100 μl) then incubated at 50°C to dissolve the gel. After dissolving the gel completely 1 volume of isopropanol was added, mixed and then the sample bound to

the QIAquick column by centrifuging at 12,000 rpm for 1 minute. The flow-through from the column was discarded and the column washed with 750 µl of buffer PE, by centrifugation at 13,000 rpm for 1 minute. The flow through was again discarded and the column spun for a further 1 minute at 13,000 rpm to ensure that buffer PE is washed from the column so the salts of this buffer will not interfere with later steps. The column was then placed into a clean 1.5ml microcentrifuge tube, to collect the purified DNA. To eluate the purified DNA from the column, 30-50 µl of dH20 was added and left for 1 minute before centrifuging at 13,000 rpm for 1 minute. The purified DNA eluated from the column was then stored at -20°C.

### 2.1.3 BLUNT ENDING OF DNA.

Klenow enzyme was used to fill in recessed 3' terminal created by digestion of DNA with restriction enzyme. 0.5 µg of DNA was blunt ended with 2.5 units of Klenow enzyme (Boehringer Mannheim), 5x buffer L, (10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.5), along with 2 mM of each dNTP. The reaction was incubated for 30 minutes at R.T. and then stopped by adding 1 µl of 0.5 M EDTA.

### 2.1.4 REMOVAL OF 5' PHOSPHATE GROUPS FROM DNA.

The dephosphorylation of DNA for the purposes of minimising recircularisation of vector DNA was accomplished by treating the restricted DNA with calf intestinal phosphate (CIP) (Boehringer Mannheim). 10-20 µg of plasmid DNA was digested with a two-threefold excess of restriction enzyme until digestion was complete. The sample was then extracted with an equal volume of phenol:chloroform and the remainder of the DNA was then precipitated by addition of 2.5 volumes of ethanol. After 20 minutes on ice the DNA was recovered by centrifugation for 30 minutes at 12,000 rpm. The DNA was then resuspended in 90µl of T.E. buffer (pH 8.0). 10 µl of 10x CIP dephosphorylation buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris-HCl pH 8.3) was added to the sample along

with 2 units of CIP enzyme. This was incubated at 37°C for 60 minutes. After incubation EDTA (pH 8.0) was added to a final concentration of 5mM and the reaction heated to 85°C for 15 minutes to inactivate CIP. The solution was then extracted with phenol and phenol:chloroform. DNA was then precipitated by addition of 0.1 volumes of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol. The DNA was recovered by centrifugation at 12,000 rpm. The pellet was washed in 70 % ethanol, air dried and the DNA resuspended in T.E. buffer (pH 8.0).

### 2.1.5 LIGATION OF COHESIVE AND BLUND TERMINI.

Rapid DNA Ligation kit was used as follows: Insert DNA and vector DNA were mixed on ice normally in a ratio 1:3 vector to insert. 100 to 150 ng of DNA mix was used, and made up to 10 µl with 1x DNA dilution buffer (Boehringer Mannheim). After mix thoroughly 10 µl of T4 DNA ligation buffer and 1 µl of T4 DNA Ligase were added to this reaction. The ligation reaction was mixed thoroughly again and incubated at R.T. for 5 minutes and then 5 µl used to transform *E.coli* DH5 α competent cells (see section 2.1.6). In blunt-end ligations ~200 ng of DNA was used and incubation time extended to 30 minutes.

### 2.1.6 TRANSFORMATION OF COMPETENT CELLS WITH PLASMID DNA.

An aliquot of competent cells (*E.coli* DH 5 α purchased from Life Technologies, Inc.) was thawed on ice, and then the transforming DNA (usually 5 µl of the ligation reaction) was added, mixed with 50 µl of the cells and left on ice for 30 minutes. This was then incubated at 42°C for 45 seconds before being placed back on ice for a further 2 minutes. The cells were swiftly mixed with 1 ml of L-broth, transferred to a microfuge tube and left at 37°C for 60 minutes. After the incubation step, the cells are centrifuged for 1 minute at R.T. followed by resuspension of the pellet in approximately 200 µl of medium. This is then pippetted onto the surface of the L-agar, and is spread by a sterile bent glass rod until the

liquid has thoroughly soaked into the agar. The plates are then inverted and incubated for 12-16 hours at 37°C.

### 2.1.7 PREPARATION OF AGAR PLATES.

LB bottom (1 % w/v Tryptone, 0.5 % w/v yeast extract, 1.5 % w/v agar, 0.2 % w/v $MgCl_2$ x $6H_2O$, 0.1 M NaCl) was melted and cooled to 37°C before adding (50 μg/ml) ampicillin. Agar plates were poured using aseptic technique and allowed to set. Unused plates could be stored for approximately 2 weeks at 4°C.

### 2.1.8 SMALL SCALE PREPARATION OF PLASMID DNA.

The desired colony on an agar plate was used to inoculate 5ml of LB-media plus antibiotic and was grown at 37°C overnight with continuous shaking at 250 rpm. 1.5ml of each culture were decanted into eppendorf tubes and centrifuged at 13,000 rpm for 10 seconds to pellet the cells. After removal of the supernatant the pellet was then resuspended in 200 μl of solution I (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, and 2 mg/ml fresh made lysozyme) by vortexing. Next, 200 μl of freshly prepared solution II (1 % SDS, 0.2 M NaOH) was added, mixed gently by inverting. This step is required for bacterial cell wall lysis and for DNA denaturation. To neutralise, 200 μl of 3 M KAc (pH 4.8) was then added mixed thoroughly as before and left on ice for 10 minutes. The tube was then centrifuged for 5 minutes at R.T. at 13,000 rpm. The supernatant was retained and the cellular debris discarded. The DNA was then precipitated, by adding 600 μl of 100 % isopropanol and storing on ice for 10 minutes. The precipitate was spun for 8 minutes at 13,000 rpm at 4°C, and the supernatant discarded. The pellet was then washed briefly in 500 μl of 70 % ethanol, re-spun at 13,000 rpm for 5 minutes at 4°C and dried to be then resuspended in appropriate volume of buffer T.E. supplemented with DNase-free pancreatic RNase (20 μg/ml).

### 2.1.9 LARGE SCALE PREPARATION OF DNA.

Plasmid DNA was purified in large scale using the Qiagen Plasmid Kit (MAXI 25). The Qiagen plasmid purification protocol is based on a modified alkaline lysis procedure followed by binding of plasmid DNA to Qiagen anion-exchange resin under appropriate low salt and pH conditions. RNA, proteins and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA is eluted in high salt buffer then concentrated and desalted by isopropanol precipitation.

The selected bacterial colony was grown overnight at 37°C in 200 ml standard LB medium plus the appropriate antibiotic in a shaking incubator (220 rpm). The culture was then centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatant was drained by inverting the open centrifuge tube. The bacterial pellet was then completely resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). This was followed by addition of 10 ml of Buffer P2 (200 mM NaOH, 1.0 % SDS). After gentle mixing the bacteria were left to lyse the cells at R.T. for 5 minutes. Subsequently, 10 ml of chilled Buffer P3 (3.0 M potassium acetate, pH 5.5) were added, the solution mixed by inversion for 5-6 times and then incubated on ice for 20 minutes. The lysate was centrifuged in a non glass tube at over 15,000 rpm for 30 minutes at 4°C. The clear supernatant was removed promptly and filtered over a prewetted folded filter.

Qiagen-tip columns (one per each culture) were equilibrated by applying 10 ml of Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15 % ethanol, 0.15 % Triton X-100), which was allowed to drain by gravity. The filtered supernatant was then loaded onto the column and allowed to enter the resin by gravity flow. The Qiagen-tip column was washed twice with 30 ml of Buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15 % ethanol). Finally the plasmid DNA was eluted by gravity from the column with 15 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15 % ethanol). The DNA was then precipitated with 10.5 ml (0.7 volumes) of isopropanol at R.T. The precipitate was centrifuged immediately at 11,000 rpm for 30 minutes at 4°C. Supernatant was carefully removed and DNA was washed with 5 ml of 70 %

ethanol, allowed to air dry for 5 minutes and resuspended in a suitable volume of $H_2O$.

### 2.1.10 PURIFICATION OF DNA.

**a) Phenol/chloroform extraction.**

To remove proteins from DNA samples, the DNA was extracted twice with a mixture containing a phenol/chloroform (1:1). Normally, 0.1 volume of the organic mixture was added to the tube, followed by a brief vortexing and then centrifugation at 11,000 rpm for 2 minutes. The upper aqueous layer was then carefully removed, avoiding the protein precipitate at the interface between the 2 layers. The DNA was then recovered by ethanol precipitation as detailed below.

**b) Ethanol precipitation.**

To concentrate DNA and remove salts 0.1 volume of 5 M sodium acetate (pH 5.5) and 2 volumes of 100 % ethanol were added to the DNA solution in a microfuge tube. The contents of the microfuge tube were mixed and chilled at -70°C for 1 hour. The tube was then centrifuged at 11,000 rpm for 15 minutes at 4°C. Supernatant was poured off and the pellet washed in 70 % ethanol, re-spun and finally resuspended in the desired volume of $dH_2O$ or buffer T.E.

### 2.1.11 CONCENTRATION OF DNA.

The DNA concentration and purity was estimated by measuring absorbance ratio at 260/280nm. In general highly pure DNA has a 260/280nm ratio >1.8, while protein contamination lowers this ratio. The quality of DNA was checked by running a mini-ethidium bromide agarose gel. Typically a 5 µl aliquot of the plasmid and 5 µl loading dye (40 mM EDTA, 0.1 %SDS, 30 % w/v Ficoll and 1.2 mg/ml bromophenol blue) was loaded and run on the gel.

### 2.1.12 PURIFICATION OF TRANSGENE FRAGMENT FOR MICROINJECTION.

The 6.95 kb aP2-NTR fragment was isolated from the plasmid by double digestion *Sal* I/*Not* I and purified using a QiaQuick spin gel extraction kit as described in section 2.1.2. Approximately 5 μg of DNA was used in the digestion. The transgene was resuspended in low salt buffer (0.2 M NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA) and purified by phenol/chloroform extraction (see above 2.1.10). The concentration (60 ng/μl) was estimated by comparison with a standard of known concentration on a 1% agarose gel.

## 2.2 NUCLEIC ACID ANALYSIS.

### 2.2.1 ISOLATION OF MOUSE-TAIL GENOMIC DNA.

Five weeks old mice were anaesthetised and 1-2cm tail biopsies taken for preparation of genomic DNA according to the method described by Laird *et al.* (1991). The biopsies were incubated in 0.5 ml digestion buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 200 mM NaCl, 0.2 % SDS and fresh 100 μg Proteinase K/ml) overnight in a shaking incubator at 55°C. Tail residues were centrifuged for 5 minutes at 13,000 rpm in a microfuge at 4°C and the supernatant decanted into 0.5 ml of isopropanol, inverted several times until threads of DNA were visible. The DNA is recovered by lifting the aggregated precipitate from the solution using a disposable yellow tip. Excess of liquid is dabbed off and the DNA is dispersed in a prelabelled tube containing an appropriate volume of T.E. Complete dissolution of the DNA required incubation for a few hours at 55°C. The DNA was then stored at -20°C and used for PCR and/or Southern blotting.

### 2.2.2 ISOLATION OF GENOMIC DNA FROM LIVER.

Liver samples that were kept in liquid nitrogen were thawed into a Dounce homogeniser with 3ml of ice cold RSBE (10 mM Tris, 10 mM NaCl, 2 mM EDTA

pH 7.4) plus 0.5 % NP40 and homogenised with as few strokes as possible. Homogenates were transferred to a 13 ml plastic Sorval tube, spun down at 4,500 rpm 5 minutes at 4°C. Supernatant was then poured off and the pellet resuspended in 3 ml of RSBE plus NP40 as above. The samples were re-spun and the pellet washed 3 times with the same solution as above. Then, the pellet was resuspended in 3 ml of RSBE, resuspended completely before 3 ml of SNET (1 % SDS, 0.6 M NaCl, 10 mM EDTA, 20 mM Tris) were added. 40 µl of Proteinase K (10 mg/ml) were added, mixed by inversion and incubated at 37°C overnight or at 55°C for 2 hours. Proteinased samples were extracted with an equal volume of phenol:chloroform (1:1), and the final extract precipitated with an equal volume of isopropanol. The visible clump of white strands of DNA was pulled out of the solution using a heat sealed pasteur pipette. DNA was dipped/washed into 75 % ethanol and allowed to air dry before being resuspended in approximately 1 ml of T.E. buffer.

### 2.2.3 ISOLATION OF GENOMIC DNA FOR DNA FRAGMENTATION ASSAYS.

Cells were harvested by centrifugation at 200 x G for 10 minutes. The pellet was lysed with 0.4 ml hypotonic lysing buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2 % Triton-X 100, and the lysates were centrifuged at 13,000 x G for 10 minutes to separate intact from fragmented chromatin. The supernatant, containing fragmented DNA, was placed in a separate microfuge tube. The pellet was digested as described in section 2.2.1 (above) and the supernatant was precipitated overnight at -20°C in 50 % isopropanol and 0.5 M NaCl. The precipitate was pelleted by centrifugation at 13,000 x G for 10 minutes, air-dried, and resuspended in appropriate volume of T.E.

### 2.2.4 ISOLATION OF TOTAL RNA FROM CULTURED CELLS.

The extraction of total RNA from cultured cells was performed using RNazolB (Biogenesis, Poole, UK), which promotes the formation of complexes of

RNA with guanidinium and water molecules and abolishes hydrophilic interactions of DNA and proteins. During the extraction step the RNA remains in the aqueous phase, separated from DNA and proteins. Cultured cells (confluent flasks with approximately 1x $10^7$ cells) were washed once in PBS and harvested by trypsinisation. The cells were pelleted by centrifugation at 1,000 rpm for 5 minutes and the supernatant removed. The cells were then lysed by resuspending in 1ml of RNAzol B. The RNAzol B solution containing the resuspended cultured cells was transferred to a screw-cap microfuge containing 100µl of chloroform. The tube was inverted repeatedly for 15 seconds and placed on ice for 15 minutes, then centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube where it was washed twice in 70 % ethanol and then vacuum-dried. The pellet was then left to dissolve overnight in 50-100 µl of DEPC treated $dH_2O$ at 4°C.

The concentration of RNA was measured by reading the absorbance of 1 µl in 200 µl dilution in DEPC treated $dH_2O$ at 260 nm wavelengths. The concentration was then calculated in mg/ml by multiplying the $A_{260}$ nm of the diluted sample by 10. The ratio A260:A280, indicating a measure of the quality of RNA, was considered optimal within a range of 1.6 to 2.0.

### 2.2.5 ISOLATION OF TOTAL RNA FROM TISSUES.

Epididymal fat pads as well as other tissues were removed and snap frozen in liquid nitrogen. Total RNA was isolated using the RNAzol B method (Biogenesis, Poole, UK) as described above for cells.

## 2.3 ELECTROPHORETIC TECHNIQUES.

### 2.3.1 AGAROSE GEL ELECTROPHORESIS OF GENOMIC DNA.

DNA molecules were separated according to their size on horizontal agarose gels. The percentage of agarose used to make the gel was dictated by the size of the

range of the DNA molecules to be resolved. Digested genomic DNA or plasmid DNA were commonly run on 1 % agarose gels, whereas smaller fragments were run on 1.5-4 % agarose gels. All agarose gels were made and run in 1x TAE (40 mM Tris-acetate, 2.5 mM EDTA pH 7.7). To stain the DNA, ethidium bromide was added to a final concentration of 1 μg/ml. The gel was poured and allowed to set before submerging in 1x TAE running buffer. Blue/Orange loading dye was added to the DNA at 1 μl per 10 μl sample volume prior to loading the sample onto the gel. Either minigels (30 ml agarose solution) or midi gels (120 ml agarose solution) were run at 40-100V depending on resolution and run-time required. DNA fragments were visualised on a mid-range UV transiluminator and photographed using video copy processor (Mitsubishi).

### 2.3.2 FORMALDEHYDE GEL ELECTROPHORESIS OF TOTAL RNA.

RNA molecules were separated by electrophoresis on 1.5 % denaturating MOPS-formaldehyde agarose gels (0.02 M 3-N-[morpholinol] propanesulfonic acid, 5 mM sodium acetate containing 6.8 % (v/v) formaldehyde essentially as described by Sambrook *et al.* (1989). Agarose was dissolved in $dH_2O$, left to cool to 50°C, then the formaldehyde solution and 1x MOPS were added. The gels were left to set and then loaded in a fume hood. The RNA sample to be loaded onto the gel (5-20 μg) was made up to the required volume containing 50 % formamide, 1x MOPS and 8 % formaldehyde. Prior to loading the samples 1x loading buffer was added. Gels were run at 40 V over night. The gel was then stained with ethidium bromide and photographed as for DNA.

### 2.3.3 DENATURING (SDS/PAGE) POLYACRYLAMIDE GEL ELECTROPHORESIS.

One dimensional SDS-PAGE gel electrophoresis was performed as described by Laemmli, (1970). Using a premixed polyacrylamide solution (30 %) supplied by Scotlabs, a 12 % running gel with 4 % stacking gel was cast. Protein samples in

electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10 % Glycerol, 2 % SDS, 5 % β-Mercaptoethanol) were boiled for 5 minutes to ensure complete denaturation of the proteins before loading on the gel. Gels were electrophoresed at a constant current of 32 mA for about 1 hour (stacking gel) and 48 mM for 5 hours (separating gel) in running buffer (25 mM Tris, 192 mM glycine, 0,1 % SDS, pH 8.3).

## 2.4 NUCLEIC ACID TRANSFERS.

### 2.4.1 TRANSFER OF DNA FROM AGAROSE GELS TO NYLON MEMBRANE.

DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes (Zeta-Probe, BioRad) using the blotting method essentially described by Southern, (1975). The DNA (fragments >8 kb) was firstly depurinated by gently shaking in two changes of 0.2 N HCl for 15 minutes. Then, the gel was soaked in 1.5 M NaCl, 0.5 M NaOH for 2x 20 minutes, and then in 1 M $NH_4OAC$, 0.02 M NaOH for 2x 30 minutes. The gel was placed on a wick consisting of two sheets of Whatman 3 MM paper on a glass plate and soaked in 10x SSC (3 M NaCl, 0.3 M sodium citrate). A piece of membrane cut to the size of the gel was prewetted in 2x SSC before being placed onto the gel. This was followed by two pieces of Whatman 3MM paper cut to the same size and prewetted in 2x SSC. Air bubbles were removed using a sterile plastic pipette. Paper towels were placed on top of the 3 MM paper followed by a glass plate and finally a weight (approximately 0.5 kg) and the DNA allowed to transfer to the membrane by capillary action as the transfer buffer ($NH_4OAc$/NaOH) is drawn through the paper towels. After 3 hours the wet paper towels nearest to the gel were replaced by dry towels and the transfer allowed proceeding for approximately 20 hours in total. The membrane was removed, rinsed in 2x SSC, air-dried and the DNA cross-linked onto the membrane using an automatic UV-linker (Stratagene).

### 2.4.2 TRANSFER OF RNA FROM FORMALDEHYDE GELS TO NYLON MEMBRANES.

A similar procedure as above was used to transfer RNA but depurination, denaturation and neutralisation steps were omitted. The transfer of the RNA was done with 10x SSC, Boehringer membrane and Whatman paper were prewetted with sterile DEPC-treated water.

## 2.5 RADIOLABELLING OF DNA.

### 2.5.1 RANDOM PRIMING OF DNA PROBES.

This method is adapted from Feiberg and Vogelstein (1984). DNA labelling with [$\alpha$-$^{32}$P]-dCTP involves random priming from hexanucleotides and then polymerisation along the DNA strand by Klenow fragment of *E.coli* polymerase I. The DNA (1-25 ng) was initially denatured by heating to 100°C for 5 minutes. DNA was then kept on ice to prevent re-annealing of the denatured DNA strands. The labelling reaction was carried out using a Hi-Prime kit (Boehringer Mannheim): 11 µl of DNA plus water was mixed with 5 µl of 10 µCi/µl [$\alpha$-$^{32}$P]-dCTP (3000 Ci/mmol;Amersham), and 4 µl Hi-Prime (containing the enzyme, nucleotides and buffer). The reaction was then incubated at 37°C for 30 minutes, and purified through a Nick column (Pharmacia Biotech) prior to be added to the hybridisation solution.

## 2.6 HYBRIDISATION OF DNA AND RNA PROBES TO NYLON MEMBRANES.

The hybridisation conditions described by Church and Gilbert (1984) were used with some minor modifications. Membranes were prehybridised for 30 minutes with 10 mls of hybridisation solution (0.5 M phosphate buffer pH 7.2, 7 % SDS, 1 mM EDTA) in a roller oven (Techne) at 65°C. The excess probe was removed by washing with 2x SSC/0.1 % SDS for 10 minutes followed by a wash with 0.2x

SSC/0.1% SDS for 15 minutes. Membranes were wrapped in saran Wrap and either exposed to phosphorimager screen or to X-ray film (AGFA: CURIX RP1) at -70°C with intensifying screens.

## 2.7 TRANSGENE MICROINJECTION.

Approximately 10 μg of the aP2-NTR was double digested with *Sal* I/*Not* I and the transgene purified free of any plasmid sequences, which are thought to interfere with the stable integration of the transgene. The concentration of the transgene DNA was determined by agarose gel analysis comparing with a standard of known concentration and aliquots of 60 ng/μl stock prepared in T.E. buffer. The DNA was microinjected at a concentration of 1.5 ng/ml into pronuclear stages eggs from superovulated (C57Bl/6J x CBA F1) females by Roberta Wallace.

## 2.8 AMPLIFICATION OF DNA AND RNA BY THE POLYMERASE CHAIN REACTION (PCR).

### 2.8.1 GENOTYPING OF TRANSGENIC MICE BY TAIL DNA PCR.

Tail DNA samples were subjected to a PCR reaction to analyse for the presence of the transgene (*E.coli* NTR gene). The reaction tube in a total volume of 25 μl contained 2 μl of commercial 10x buffer supplied by Promega (500 mM KCl, 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 0.1 % gelatin, 1 % Triton X-100), 1 μl of each primer (see below for the sequence) at 30 pmol/μl, 2.5 μl of dNTPmix (2,5 mM stock mix) and 0.3 μl of thermostable DNA polymerase (*Thermus aquaticus*) also supplied by Promega. 1 μl of crude tail DNA was added to each tube and a known positive and negative tail sample set up alongside to act as controls for the reaction. Thermal cycling was controlled by a programmable heating block (Perkin Elmer 9700) and the following program executed:

a) Genotyping for NTR:
5'-GCAGATCAAAACGCTACTGCAATAC-3' (forward)
5'-AGCCTTTCTCTTTCAGACCAAATTC-3' (reverse)

94°C/4' followed of 30 cycles of 94°C/1', 62°C/1', 72°C/1' and a final extension of 72°C/7'.

b) Genotyping for $Lep^{ob}/Lep^{ob}$:

5' TGACCTGGAGAATCTCT 3' (forward mutant)

5' GACCTGGGAGAATCTCC 3' (forward wild type)

5' GCACATGGCTCTCTTCT 3' (reverse common for both)

94°C/4' followed of 30 cycles of 94°C/1', 58°C/1', 72°C/1' and a final extension of 72°C/7'. Annealing temperature was modified from 52°C (originally described by Anand and Chada (2000) to 58°C.

Normally, 10 µl of the amplified DNA samples was electrophoresed in a 1 % agarose gel.

### 2.8.2 AMPLIFICATION FROM RNA (RT-PCR).

Amplification of RNA was carried out using the SupersScript™ II RNase H⁻ Reverse Transcriptase and the conditions given by the provider (Gibco BRL). 1-5 µg of RNA (DNase-treated), 1 µl of Oligo $(dT)_{12-18}$ and 12 µl of DEPC-dH2O were mixed in a nuclease-free microcentrifuge tube. The mixture was then heated at 70°C for 10 minutes and quick chilled on ice. 4 µl of 5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 0.1 M DTT, and 1 µl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP) were added to the tube and incubated at 42°C for 2 minutes. 1 µl of SuperScript II (200 units) were mixed by pipetting gently up and down and the mixture incubated 50 minutes at 42°C. The reaction was then inactivated by heating the tube at 70°C for 15 minutes. Normally 10 % of this final reaction was used in a standard PCR reaction. PCR primers were chosen from published sequence data using the software Primer design. PCR conditions for each set of primers were assessed considering the temperature melting for each of the primers.

Primer sequences and parameter of the PCR reactions:

a) Pref1:

5' GATTCTGCGAGGCTGACAAT 3' (forward)

5' ATCGTTCTCGCATGGGTTAG 3' (reverse)

94°C/4' followed of 30 cycles of 94°C/1', 60°C/1', 72°C/1' and a final extension of 72°C/7'.

d) Adipsin:

5' ATGACGACTCTGTGCAGGTG 3' (forward)

5' GTATAGACGCCCGGCTTTTT 3' (reverse)

94°C/4' followed of 30 cycles of 94°C/1', 61°C/1', 72°C/1' and a final extension of 72°C/7'.

e) RAIDD Endogenous/exogenous:

5' AAACGGTACGTGGTCGCAGA 3' (forward)

5' GCGAATGCACGTTGTGGGGA 3' (reverse)

94°C/4' followed of 30 cycles of 94°C/1', 63°C/1', 72°C/1' and a final extension of 72°C/7'.

f) RAIDD Exogenous:

5' AGCACCCTCCTGTGCAG 3' (forward)

5' GCGAATGCACGTTGTGGGGA 3' (reverse)

94°C/4' followed of 30 cycles of 94°C/1', 68.5°C/1', 72°C/1' and a final extension of 72°C/7'.

## 2.9 CULTURING OF 3T3L1 PREADIPOCYTE CELL LINE.

### 2.9.1 CULTURE MEDIUM.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing:

-10 %FCS

-L-glutamine (final conc. 50 μg/ml)

-Penicillin/Streptomycin (final conc. 10,000 U/ml of each)

-Non-essential aminoacids (5.6 ml of 100 X stock)

Medium was changed every 3 days when cells were subconfluent and every 2 days when cells were confluent.

### 2.9.2 FREEZING AND RESUSCITATION OF 3T3L1 CELLS.

Cells were harvested from either a T-25cm$^2$ or T-80 cm$^2$ flasks as described above and resuspended in 0.5 ml of complete medium and placed on ice. An equal volume of ice-cold freezing mix was added drop-wise with continual mixing of the contents of the tube.

Freezing mix:

60 % (v/v) Complete medium

20 % (v/v) FCS

20 % (v/v) dimethyl sulfoxide (Sigma)

The mixture was dispensed into 1 ml aliquots into pre-chilled screw cap tubes, placed in a polystyrene box and incubated at -20°C overnight before transferring to liquid nitrogen for long term storage.

To resuscitate the cells, the vial was transferred from the liquid nitrogen to a beaker of water preheated to 37°C and agitated until the mixture thawed. The contents of the tube were added to 10 ml of complete medium and the tube rinsed with 1 ml of medium. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes, and then the supernatant removed. The pellet was resuspended in 5 ml of complete medium, with a glass pipette to help formation of single cell suspensions. The resuspended cells were transferred to a T-25cm$^2$ flask and placed in a $CO_2$ incubator at 37°C.

### 2.9.3 INDUCTION OF DIFFERENTIATION OF 3T3L1 CELLS.

The protocol used to differentiate these cells was basically as described by Student *et al.* (1980). Two days post-confluent cells were induced to differentiate in medium containing the following cocktail of hormones:

-Insulin (final conc. 1.7 μM).

-Dexamethasone (final conc. 1 μM).

-IbuMeXan (final conc. 0.5 mM).

Cells were incubated in this medium for two days after which time medium was replaced with DMEM supplemented only with insulin and 10 % FCS.

### 2.9.4 TRANSFECTION OF 3T3L1 CELLS.

Cells were transfected using the Calcium phosphate protocol as described in Sambrook *et al.* (1989). The day before of the transfection 5 $x10^5$ 3T3L1 cells were seeded in a T-80 flask (80 $cm^2$) with DMEM complete medium and incubated at 37°C, 5 % $CO_2$. On the day of the transfection the medium was changed to 15 ml of fresh medium and the cells incubated for 3 hours at 37°C, 5 % $CO_2$. Whilst the cells were incubating, $CaCl_2$, 2x HBS and the DNA solutions were left to defrost at room temperature. One hour before the transfection 0.5 ml aliquots of 2x HBS were prepared in bijoux's. In a separate tube the plasmid DNA's were mixed to $CaCl_2$ and made up to 0.5 ml with $dH_2O$, to give a final concentration of 20 μg of DNA and 250 mM $CaCl_2$. The DNA-calcium chloride solution was then added drop-wise to the 2x HBS and left at R.T. for 20 minutes. After the three hours incubation period, 1 ml of the DNA-calcium chloride/HBS solution was added in a drop-wise fashion to the cells, and gently shaken to mix. The precipitated DNA was left on the cells for four hours in the incubator at 37°C, 5 % $CO_2$. After 48 hours the medium was changed to selection medium containing G-418 (400 μg/ml). G-418 selection medium was applied for a further 12 days, with media being changed every second day and the cells incubated at 37°C, 5 % $CO_2$. After 14 days of selection, individual colonies were picked up and transferred to small 6 well dishes. Transformed cells were cultured in complete medium without the drug and each of the clones expanded and kept in liquid nitrogen for future analysis.

## 2.10 PROTEIN EXTRACTION AND ANALYSIS.

### 2.10.1 PROTEIN EXTRACTION FROM CELLS AND TISSUES.

Medium was removed from the cells and the flasks or dishes were incubated on ice for 5 minutes. Cells were washed twice with ice cold PBS and 1 ml of lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Nonidet-P40, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.057 mM PMSF (phenylmethylsulfonyl fluoride) was added to the cells in a 75 $cm^2$ flask. The flask was incubated on ice for 30 minutes and the cells were scrapped and transferred into a 1.5 ml eppendorf tube. After centrifugation of the cells for 5 minutes at 4°C, the supernatant was transferred in aliquots of 100 µl to ice cold eppendorf tubes, and these were kept for long storage at -70°C till analysis. The procedure for tissues was basically the same with the difference that tissues had to be homogenised in the lysis buffer before proceeding as described above for cells.

### 2.10.2 ESTIMATION OF PROTEIN CONCENTRATION.

Protein concentration was measured using the PIERCE detection system and the standard protocol. Protein standards were prepared in the range 200 µg/ml-1200 µg/ml by diluting the bovine serum albumin stock (2 mg/ml) provided by the manufacturers and a blank prepared with no protein. 10 µl of the nuclear extracts were diluted 10 fold with $ddH_2O$ to make a total volume of 100 µl. Then, 0.1 ml of each of the standards and the samples were pippetted into a fresh tube and 2.0 ml of Working Reagent added to each tube, mixed and incubated at 37°C for 30 minutes. During the incubation time there is a colour change from pale green to purple due to the reaction of protein with $Cu^{2+}$ in an alkaline medium to yield $Cu^{1+}$ which when complexed with bicinchoninic acid (BCA) gives a purple reaction product which exhibits strong absorbance at wavelength 562 nm.

The absorbances at wavelength 562 nm of the standard (blank corrected) were plotted against protein concentrations. Using this standard curve the protein concentration was determined.

### 2.10.3 WESTERN BLOTTING.

One-dimensional SDS-PAGE was performed to detect the NTR protein. Proteins were transferred to nitrocellulose at 0.8 mA/cm2 for 1 hour using a semi-dry Electro blotter (Khyse-Anderson, 1984). To block non-specific protein, the membrane was incubated in a solution of TBS-T (Tris buffer saline/Tween) containing 3 % BSA for 1 hour at R.T. Then, nitrocellulose membrane was exposed to a polyclonal anti-NTR antibody diluted in 1:1500 in blocking solution containing 1 % BSA for 1.5 hours at R.T. The membrane was then washed 5x with TBS/Tween at 5 minutes intervals (R.T.). The primary antibody was visualised using peroxidase-conjugated HRP-anti-rabbit IgG secondary antibody (1:5000 dilution in TBS-T/1% BSA) for 1 hour at R.T. and ECL detection reagents (Amersham).

## 2.11 HISTOLOGY AND IMMUNOCYTOCHEMISTRY.

### 2.11.1 PREPARATION OF PARAFFIN TISSUE SECTIONS FOR STAINING.

Typically the left epididymal fat pads (male) and the left parametrial fat pads (female) alongside with skin and liver samples, were removed for analysis from normal and transgenic C57BL6xCBA mice. The tissues were fixed in NBF (neutral buffered formalin) for at least 24 hours before embedding in paraffin using standard procedures. 5-8 μm sections were cut from blocks using a Microm (HM 325) Microtome. The sections were floated in a 40°C water bath and collected on glass slides.

The sections were dewaxed and rehydrated in Coplin jars using standard procedures. Briefly, sections were dewaxed in two changes of xylene and rehydrated through graded alcohol 100 %, 90 %, 80 %, 70 % ethanol/dd$H_2O$ (v/v). Finally the sections were washed in running tap water before staining procedures were carried out.

### 2.11.2 HISTOLOGICAL STAINING.

Mayer's haematoxylin-eosin staining were routinely performed to examine the morphology of the tissues. The sections were placed in Mayer's haematoxylin

solution (BDH) for 5 minutes and washed in running water for 3 minutes. The sections were then counterstained for 2 minutes with eosin (BDH), rinsed in running tap water for 30 seconds and dehydrated through graded alcohols, cleared in Kem sol and mounted in DPX mounting medium.

### 2.11.3 APOPTOSIS STAINING.

*In situ* apoptosis was detected using the apoTag kit. The test works by linking digoxigenin-nucleotides to DNA 3'OH ends, which are generated during apoptosis, using terminal deoxynucleotidyl transferase (TdT). Either dewaxed sections or fixed cells were pretreated with proteinase K (20 μg/ml) for 15 minutes at R.T. in a coplin jar or directly on the slide. Endogenous peroxidase was blocked with 3 % (v/v) hydrogen peroxide for 5 minutes at R.T. After treatment of the sections with the equilibration buffer the samples were incubated with TdT enzyme in a humidified chamber at 37°C for 1 hour. Anti-digoxigenin peroxidase conjugate was applied directly to the slides and incubated in a humidified chamber for 30 minutes at R.T. The colour was developed by incubation with a DAB/$H_2O_2$ solution for 10 minutes. Sections were counterstained with methyl green, washed in 3 changes of water, then washed in 3 changes of n-butanol, dehydrated in Kem sol, mounted in DPX and visualised by light microscopy.

### 2.11.4 IMMUNOCYTOCHEMISTRY STAINING.

For immunocytochemistry dewaxed sections were pretreated with 0.1 % (w/v) trypsin in PBS for 10 minutes at 37°C for antigen retrieval. Endogenous peroxidase was blocked with 1 % (v/v) hydrogen peroxide. After blocking non-specific antibody binding in 5 % (v/v) normal swine serum (Dako, Glostrup, Denmark) the sections were incubated for 1.5 hours at 37°C with the primary antibody (anti-NTR) diluted in blocking solution (1:300). After washing in PBS, sections were incubated with the secondary antibody for 1 hour at R.T. (1:200 swine anti-rabbit Ig biotinylated antibody (Dako; E0353). Sections were washed in PBS

and incubated with streptavidin-peroxidase conjugate (Zymed, San Francisco, CA, USA) at 1:2000 for 30 minutes at R.T. in the dark. The colour was developed by incubation with a DAB/$H_2O_2$ solution for 10 minutes. Sections were counterstained with haematoxylin, dehydrated and mounted in DPX and visualised by light microscopy.

## 2.12 CARCASS ANALYSIS.

Roslin Nutrition carried out the carcass analysis, and basically the protocol consisted in to dry the carcasses to constant weight at 65°C. Whole body fat was measured by Soxhlet extraction of the lipids in the dried carcasses using carbon tetrachloride as solvent. Fat mass is then calculated as the difference between dry weight before and after chloroform extraction. Lean body mass is carcass weight minus fat mass.

## 2.13 BLOOD ANALYSIS.

Animals were sacrificed by cervical dislocation between 15.00 and 18.00 hours, and trunk blood was collected. Serum Insulin and plasma leptin levels were determined by using radioimmunoassay kits with rat insulin and recombinant leptin respectively, as standards (Linco Research Immunoassay, St. Charles, MO). Plasma triglycerides, free fatty acids and β-hydroxybutyrate levels were assayed with commercially available kits (Sigma and Boehringer Mannheim). Whole blood glucose level was measured by using the One-Touch Profile glucose meter (Lifescan, Milipitas, CA).

## 2.14 STATISTICAL ANALYSIS.

ANOVA one way test and Student *t* test were use for statistical evaluation using the MINITAB program, with $P<0.05$ considered as significant. All results were expressed as means ± S.E.M unless indicated otherwise in the figures.

# 3T3L1: A CELL CULTURE MODEL TO TEST THE EFFICACY OF CB1954 MEDIATED ABLATION IN ADIPOCYTES *IN VITRO*.

## 3.1 BACKGROUND.

There are several preadipocyte cell lines reported that differentiate *in vitro* and exhibit most of the function of adipocytes *in vivo*. 3T3L1 is one such cell line initially isolated by Green and Meuth, (1974) and later by many others. These cells were chosen in this thesis because they are the best characterised and most widely used for molecular biological studies. Prior to differentiation, the preadipocyte cell lines are morphologically similar to fibroblastic preadipose cells in the stroma of the adipose tissue (Figure 3 .1 A). When appropriately induced with hormonal agents (e.g. glucocorticoids, insulin-like growth factor-1, and cyclic AMP or factors that mimic these agents as described in Chapter 2) committed preadipocytes differentiate into adipocytes in culture. In the process, the cells lose their fibroblastic character, assume a "rounded-up" appearance and acquire the morphological and biochemical characteristics of adipocytes. The rounding up of the differentiating preadipocyte is thought to be due to changes in expression of cytoskeletal and/or extracellular proteins involved in matrix formation (Spiegelman and Farmer, 1982). Consistent with this view, the cellular content of the cytoskeletal proteins actin and tubulin decreases, and a switch in the expression of collagen types occurs, concomitant with the transition from fibroblast to adipocyte morphology. Soon after the induction of differentiation (3-4 days), many cytoplasmic triacylglycerol-containing vacuoles start to appear (Figure 3.1 D) and, after an extended period in culture, coalesce to form unilocular fat droplets, thus giving rise to the typical "signet ring" appearance of mature white adipocytes (Figure 3 .1 F).

A considerable body of evidence shows that differentiated adipocytes faithfully mimic the metabolism of adipocytes isolated from adipose tissue. Indeed, extensive biochemical analysis has revealed that the accumulation of cytoplasmic triacylglycerol is closely correlated with the coordinate expression of virtually every

enzyme of the pathways of the *novo* fatty acid and triacylglycerol biosynthesis (Cornelius *et al.* 1994). Preadipocytes in culture also acquire the proteins necessary for lipolysis of triacylglycerol, uptake and intracellular translocation of fatty acids, and regulation of these processes by lipogenic and lipolytic hormones (Student *et al.* 1980; Reed and Lane, 1980). Due to the similarities of these cells with adipocytes *in vivo* it was considered for this thesis that this *in vitro* model would provide important information regarding the behaviour of the system *in vivo*, both in terms of the expression of the *E.coli* nitroreductase gene and in terms of the sensitivity of the adipose cells to the ablation mediated by the prodrug CB1954.

This chapter describes the generation and characterisation of the preadipocyte cell line (3T3L1) expressing the *E.coli* nitroreductase gene under the control of the adipocyte specific promoter aP2. A series of experiments carried out with these cells expressing NTR demonstrated the susceptibility of these cells to the ablation mediated by the prodrug CB1954. Clones of cells stably expressing NTR were killed after treatment with the prodrug, whereas non-expressing cells were unaffected. The effect on the cell killing was directly correlated with the levels of nitroreductase expression. Furthermore, the mechanism of cell killing was found to involve apoptosis as evidenced by both DNA fragmentation, and Tunel assays. This is in agreement with the proposed mechanism of toxicity of this system through DNA-crosslinking and consequent triggering of an apoptotic response. In addition, the presence of a cell-permeable metabolite that is released to the medium triggering a bystander effect towards non-expressing adipose cells was demonstrated.

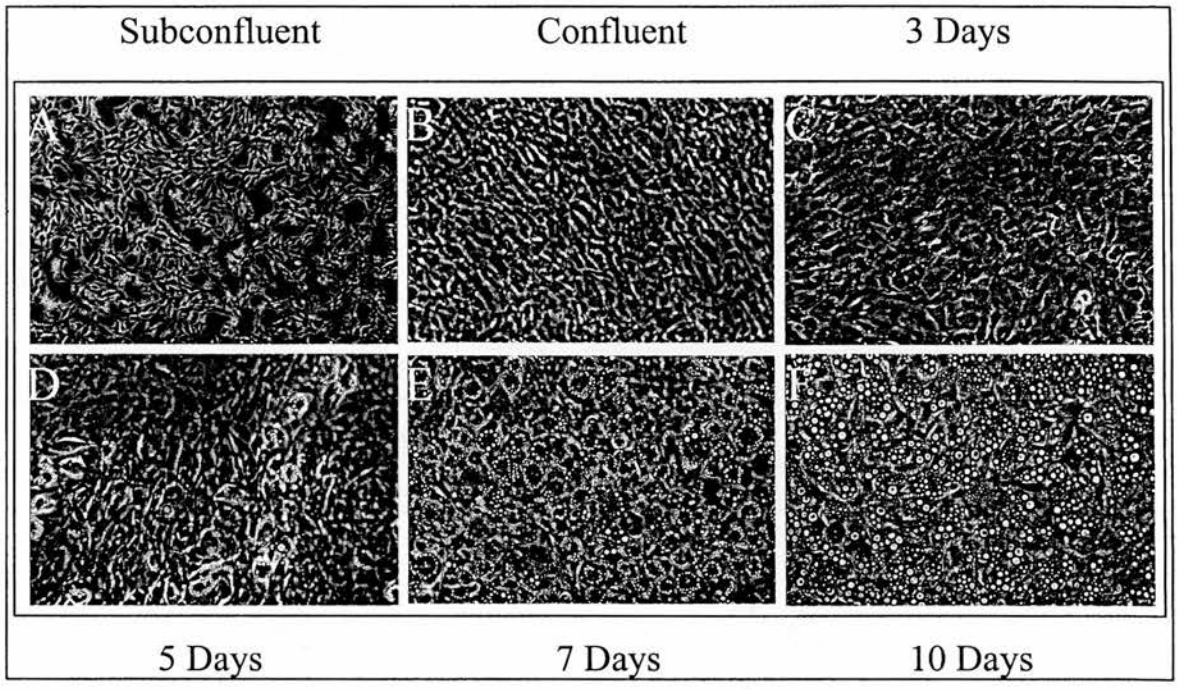



**Figure 3.1. Adipocyte Differentiation *in vitro*.**
Phase-contrast photomicrograph of the preadipocyte cell line 3T3L1 at different stages of development. A, B, C, D, E and F correspond to different stages of the adipocyte differentiation. Days after confluent correspond to days after induction to differentiate with the cocktail of hormones/steroids. Note the dramatic morphological change that has occurred after 10 days as evidenced by the lipid accumulation in the cytosol of the cells. Magnification: 100X.

## 3.2 TRANSGENE CONSTRUCTS.

### 3.2.1. GENERATION OF THE aP2-NTR CONSTRUCT.

Figure 3.2 corresponds to a diagram that summarises the cloning steps used to generate the aP2-nitroreductase (aP2-NTR) construct. A 1.55 kb fragment containing the *E.coli ntr* gene and the polyA signal from SV40 was liberated from the plasmid pWR6 (kindly provided by Dr. Wei Cui), by double digestion *Hind* III/*Xba* I (Figure 3.3 A), end filled using Klenow polymerase and ligated into the unique *Sma* I site of the aP2 promoter/enhancer plasmid (kindly provided by Dr Reed Graves). The final construct, designated paP2-NTR, was cut with a number of restriction enzymes to check for the expected banding pattern (Figure 3.3 B and Figure 3.4 for details of the expected size bands). Sequencing of the 5' junction between the NTR gene and the aP2 promoter was carried out to verify the fidelity of the ligations by an automated method using an internal primer for the NTR gene (data not shown).

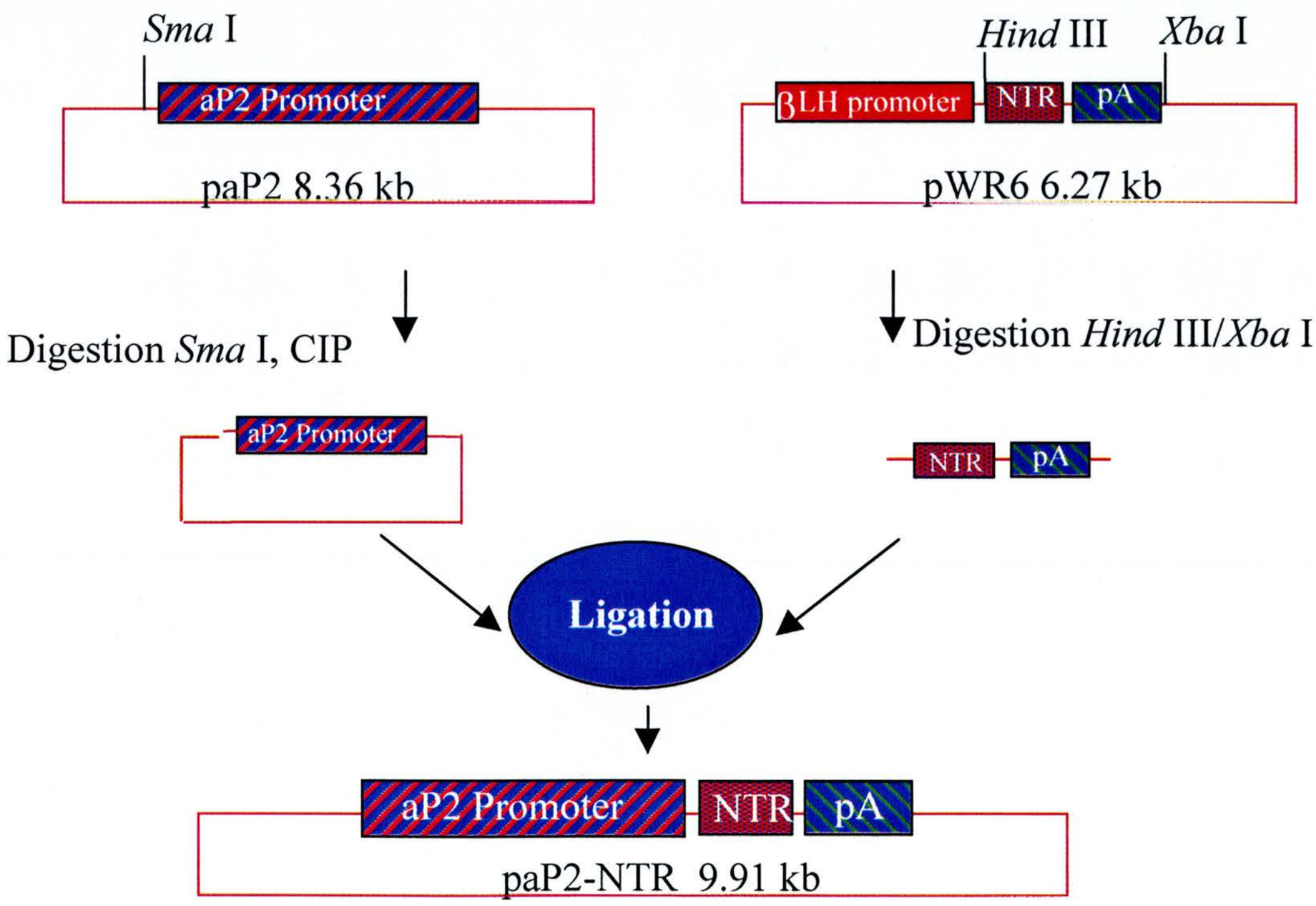



**Figure 3.2. Cloning strategy used to generate the aP2-NTR plasmid.**
1.55 kb fragment from the plasmid pWR6 was excised by double digestion *Hind* III/*Xba* I, end filled and gel purified before ligation into the unique *Sma* I site of aP2 Promoter plasmid.

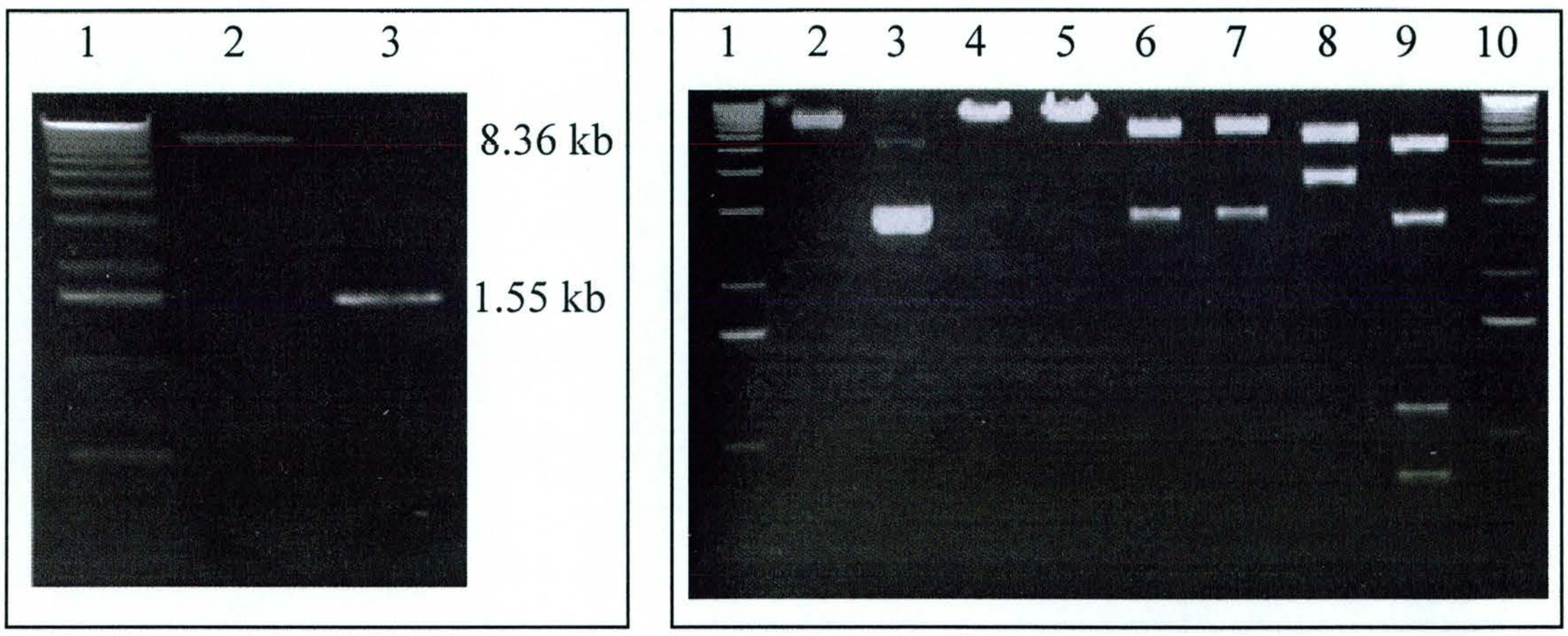



**Figure 3.3: Restriction digest of plasmids used to construct paP2-NTR.**
A) The purified 1.55 kb fragment (lane 3) from the plasmid pWR6 was cloned into the unique *Sma* I site of the aP2 plasmid (lane 2). Lane 1 is 1 kb marker.

B) A series of restriction digests from one of the putative colonies. Lanes 1 and 10, 1 kb marker. Lane 2 aP2 plasmid *Sal* I, lane 3 pSK II plasmid *Sal* I, lane 4 aP2-NTR plasmid *Sal* I, lane 5 aP2-NTR plasmid *Not* I, lane 6 aP2-NTR plasmid *Sal* I/*Not* I, lane 7 aP2-NTR plasmid *Xho* I/*Not* I, lane 8 aP2-NTR plasmid *EcoR* I and lane 9 aP2-NTR plasmid *BamH* I.


**Figure 3.4: Restriction map of the aP2-NTR plasmid.**
Probe 1 (679bp) and probe 2 (850bp) are indicated and X=*Xho* I, S=*Sal* I, E=*EcoR* I, B=*BamH* I, N=*Not* I. aP2 Promoter: 5.4 kb, NTR: 0.7 kb, pA: 0.85 kb and backbone plasmid 2.96 kb (pSK II).

| | |
|---|---|
| *Sal* I digest: | 9.91 kb |
| *Not* I digest: | 9.91 kb |
| *Sal* I/*Not* I digest: | 6.95 kb + 2.96 kb |
| *Xho* I/*Not* I digest: | 6.95 kb + 2.96 kb |
| *EcoR* I digest: | 6.21 kb + 3.70 kb |
| *BamH* I digest: | 5.26 kb + 2.70 kb + 1.1 kb + 0.85 kb |

Also in Figure 3.3.B, lanes 2 and 3 correspond to control digestions of the aP2 and pSK II plasmids, respectively. Thus, in lane 2 *Sal* I digests 8.36 kb in aP2 plasmid and in lane 3 *Sal* I digest 2.96 kb in pSK II plasmid.

### 3.2.2. GENERATION OF THE NTR-NEO CONSTRUCT.

Figure 3.5 corresponds to a diagram that summarises the cloning steps that derived in the construction of the plasmid that was used in the transfection experiments. 1.8 kb fragment containing the NEO cassette (PGK promoter, neomycin gene, and pA from SV40) was excised from the NEO plasmid (kindly provided by Dr. E. Gallagher) by digestion with *Xho* I (Figure 3.6 panel A). The aP2-NTR plasmid was also digested with *Xho* I (Figure 3.6 panel A). The 1.8 kb and the linearised plasmid fragments were gel purified and ligated to generate the NTR-NEO plasmid as shown in Figure 3.5. The final construct was cut with a panel of several restriction enzymes to check that the components of the construct and the final construct gave the expected banding pattern (Figure 3.6 B and Figure 3.7 for details of the expected size bands).

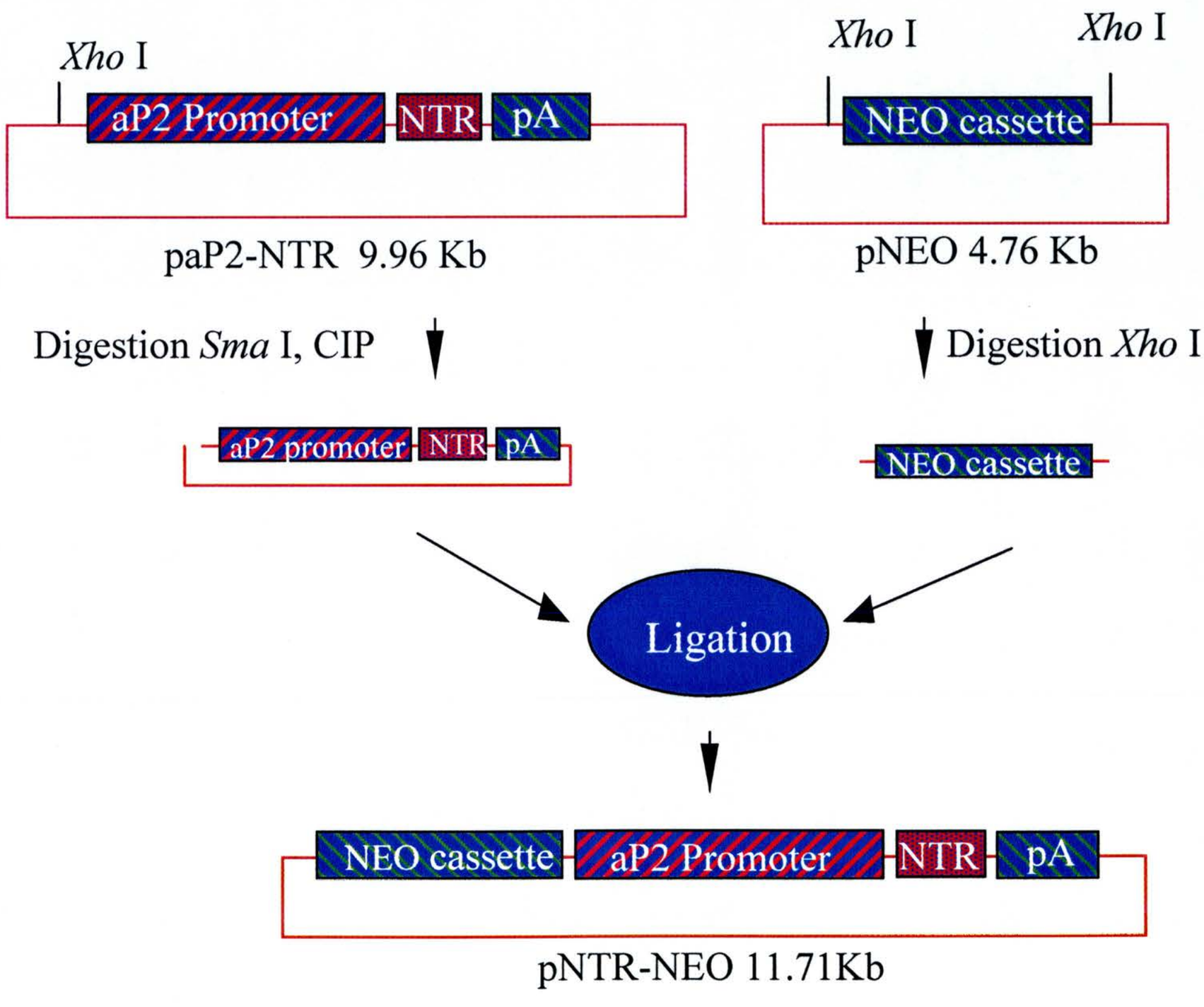



**Figure 3.5. Cloning strategy used to generate the pNTR-NEO plasmid.**
1.8 kb fragment containing the NEO cassette was excised from the plasmid pNEO, by digestion with *Xho* I and ligated into the unique *Xho* I site (CIP treated) of the aP2-NTR plasmid (described above).

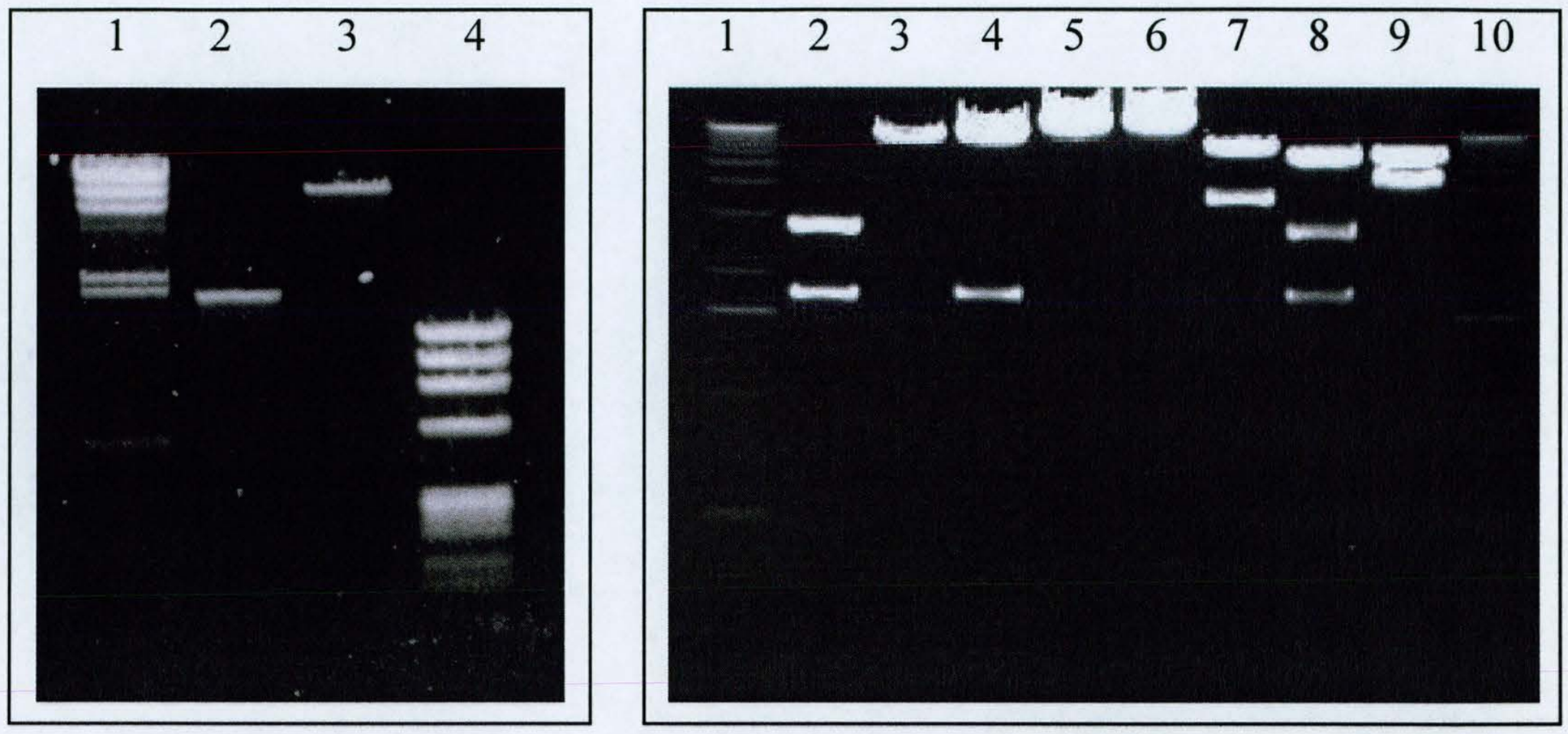



**Figure 3.6: Restriction digest of plasmids used to construct pNTR-NEO.**
A) The purified 1.8 kb fragment (lane 2) from the NEO plasmid was cloned into the unique *Xho* I site of the aP2-NTR plasmid (lane 3). Lanes 1 and 4 are λ *Hind* III and ϕ X-174 markers, respectively.

B) A series of restriction digests from one of the putative colonies. Lanes 1 and 10 1 kb marker. Lane 2 NEO plasmid *Xho* I, lane 3 aP2-NTR plasmid *Xho* I, lane 4 NTR-NEO plasmid *Xho* I, lane 5 NTR-NEO plasmid *Sal* I, lane 6 NTR-NEO plasmid *Not* I, lane 7 NTR-NEO plasmid *EcoR* I, lane 8 NTR-NEO plasmid *Xho* I/*Not* I and lane 9 NTR-NEO plasmid *Sal* I/*Not* I.

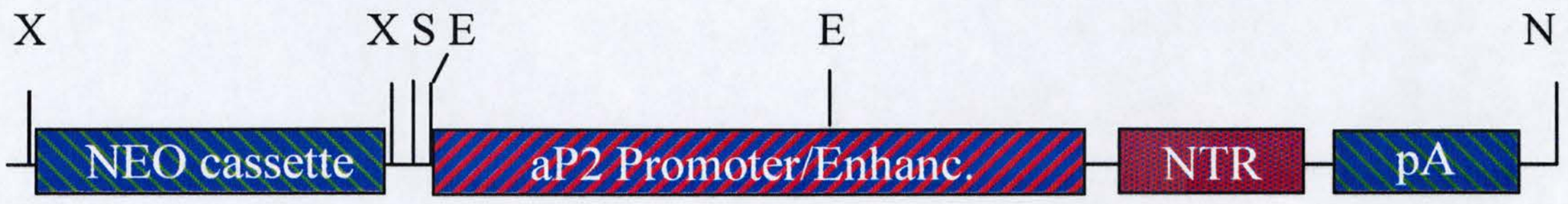



**Figure 3.7. Restriction map of the pNTR-NEO plasmid.**
NEO cassette: 1.8 kb, aP2 Promoter: 5.4 kb, NTR: 0.7 kb, pA: 0.85 kb and backbone plasmid 2.96 kb (pSK II).

| | |
|---|---|
| X=*Xho* I digest: | 1.8 kb + 9.91 kb |
| E=*EcoR* I digest: | 3.7 kb + 8.01 kb |
| Xho I/Not I digest: | 1.8 kb + 2.96 kb + 6.95 kb |
| Sal I/Not I digest: | 4.76 kb + 6.95 kb |
| S=*Sal* I digest: | 11.71 kb |
| N=*Not* I digest: | 11.71 kb |

Also in Figure 3.6.B, lanes 2 and 3 correspond to control digestions of NEO and aP2-NTR plasmids, respectively. Thus, in lane 2 *Xho* I digest 1.8 kb (Neo cassette) and 2.96 kb (backbone plasmid) bands in the NEO plasmid and in lane 3 *Xho* I digest 9.91 kb in aP2-NTR plasmid.

### 3.3 TRANSFECTION OF 3T3L1 CELLS.

3T3L1 cells were first co-transfected by calcium phosphate using both the aP2-NTR and NEO plasmids. Pools of approximately 50 colonies were analysed for the expression of the transgene by either Northern Blot or RT-PCR, after differentiation of these cells into adipocytes as described in section 2.9.3 in chapter 2. Due to the low levels of expression seen only by the latter approach (data not shown) it was decided to use a different strategy this time transfecting the cells with a plasmid containing both the aP2 driven NTR and the selectable marker neomycin. On this second strategy, cells were transfected with the NTR-NEO plasmid by calcium phosphate, and kept in 400 μg/ml of neomycin selective medium, as suggested by Hunt *et al.* (1986) for approximately 11 days (see Figure 3.8). In this way, a number of clones were isolated using cloning rings and expanded in neomycin free medium. Each of these clones was then analysed for nitroreductase expression (see below) and a stock of each was kept in liquid nitrogen for future experiments.

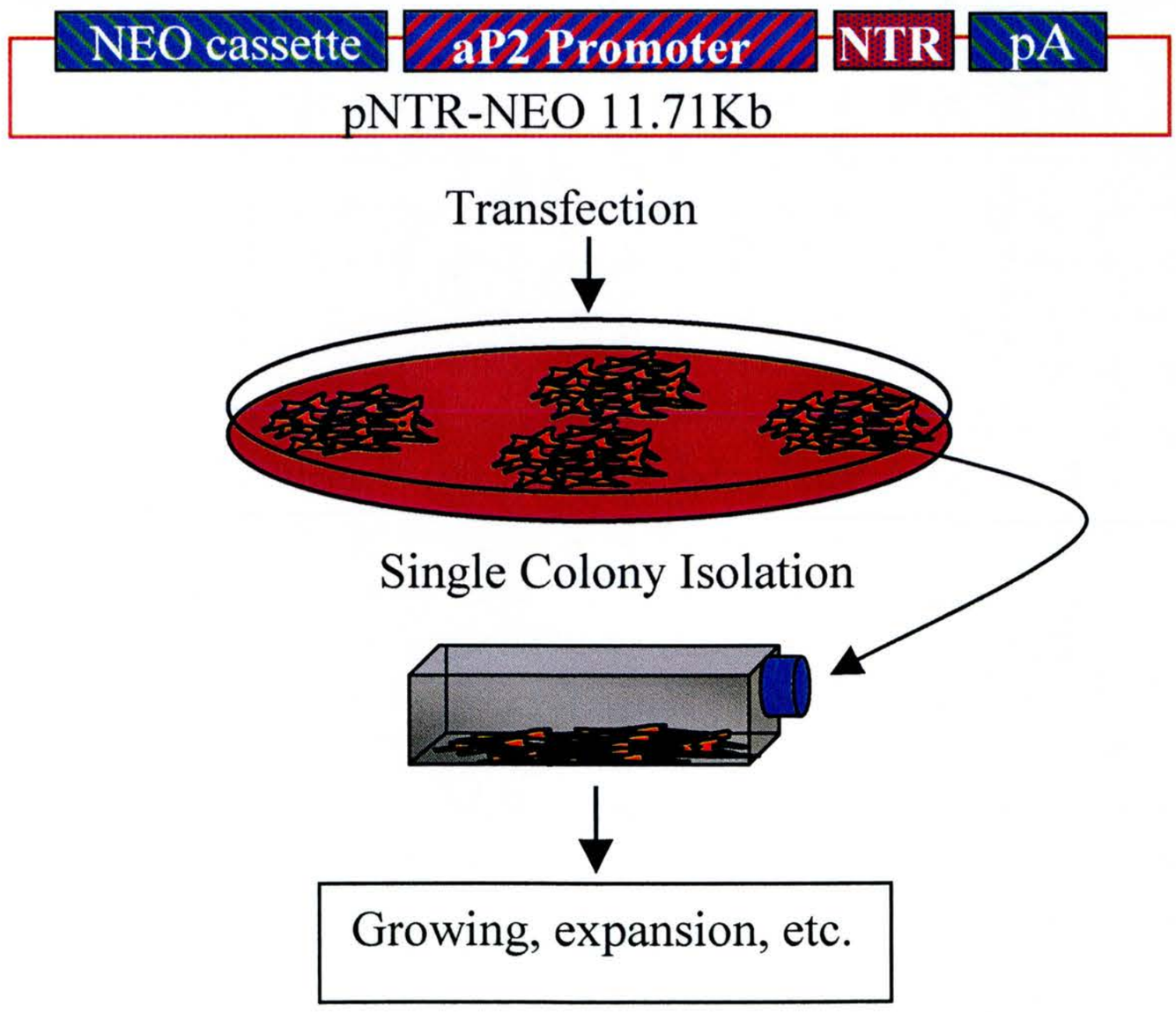



**Figure 3.8. Stable transfection strategy.**
The figure shows the strategy followed to isolate single colonies of 3T3L1 cells transfected with the plasmid NTR-NEO.

## 3.4 EXPRESSION ANALYSIS OF TRANSFECTED 3T3L1 CELLS.

Transfection of the cells by calcium phosphate using the second strategy as detailed above, allowed the isolation of clones expressing different levels of NTR after differentiation of these cells into adipocytes. Analysis of RNA carried out by either Northern blot or semi-quantitative PCR (Figures 3.9 A and B) showed that two of the clones (A and 10) had relatively high levels of nitroreductase. The rest of the clones either did not express or expressed NTR at low levels after differentiation. This data confirmed that both the plasmid and *E.coli* sequences used in the transfection are not detrimental to the survival of the cells and do not interfere with the appropriate differentiation of these cells into adipocytes.

This data also confirm that the sequence of the aP2 promoter used contains all the necessary elements to drive relatively strong levels of expression in adipocytes *in vitro*.

A)

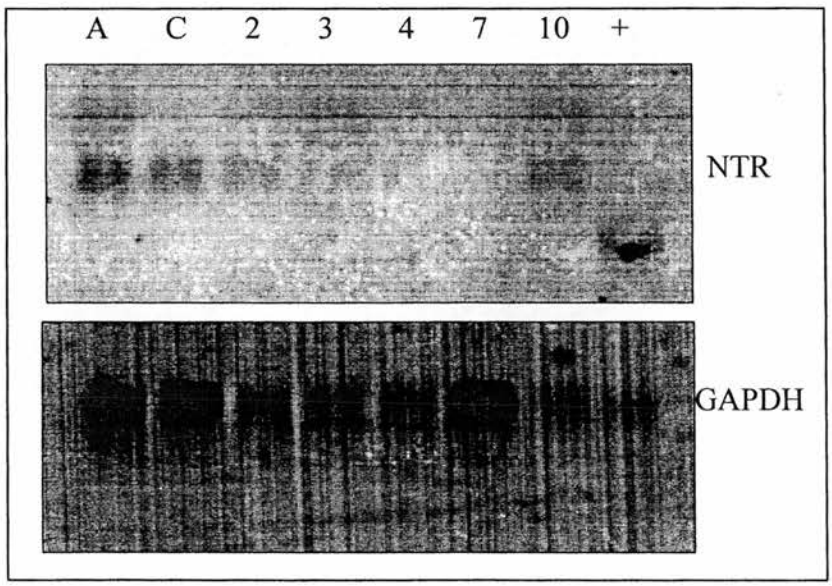
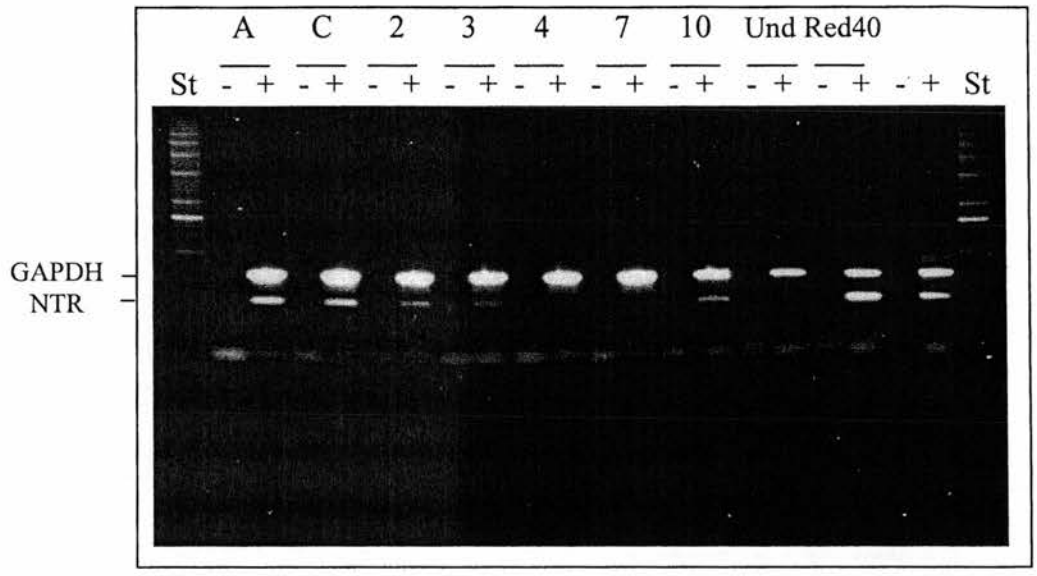



**Figure 3.9. RNA expression of NTR transgene in transfected 3T3L1 cells.**
**A) Northern blot from different clones of cells expressing the *ntr* gene.** 10μg of total RNA was run on a denaturing agarose gel and Northern blot hybridised with probe 1 (Figure 3.4) to detect the transgene (superior panel) and GAPDH probe as a loading control (lower panel).
+: Positive control, RNA from a mouse expressing NTR in mammary gland.

B)




**B) Semi-quantitative PCR from different clones of cells expressing the *ntr* gene as in A.** 3μg of total RNA was DNAse treated, reverse transcribed with oligo (dT)12-18mer (Gibco BRL) and subjected to PCR with primers for NTR and GAPDH (internal control). Reactions with (+) and without (-) reverse transcriptase are shown.
Und: Undifferentiated cells
Red 40: Positive control (RNA from a mouse expressing NTR in mammary gland).
-/+ : Negative and positive controls for the PCR reaction respectively.

### 3.5 TIME COURSE EXPRESSION OF THE TRANSGENE.

The developmental regulation of expression of nitroreductase from the aP2 promoter was studied by following the time course of RNA accumulation. Transfected cells were differentiated as described in section 2.9.3 in chapter 2 and RNA was made at different time points of the differentiation protocol. In transfected cells, both the endogenous aP2 and the exogenous NTR mRNA increased with a similar time course during differentiation and reached a plateau at the same time (Figure 3.10). As expected, both mRNA species were undetectable prior to the differentiation of the cells thus confirming the previous observations that this

promoter is active only in differentiated cells (Ross *et al.* 1990). This result indicates that the aP2 promoter sequences used in the construct contains most of the cis-regulatory elements needed for an appropriate developmental expression in adipocytes. Thus, it was concluded from these results that the exogenous gene is activated and developmentally regulated within adipocytes in a qualitatively similar fashion to the endogenous aP2 gene.

## TIME COURSE OF mRNA EXPRESSION OF BOTH ENDOGENOUS (aP2) AND EXOGENOUS (NTR) GENES.

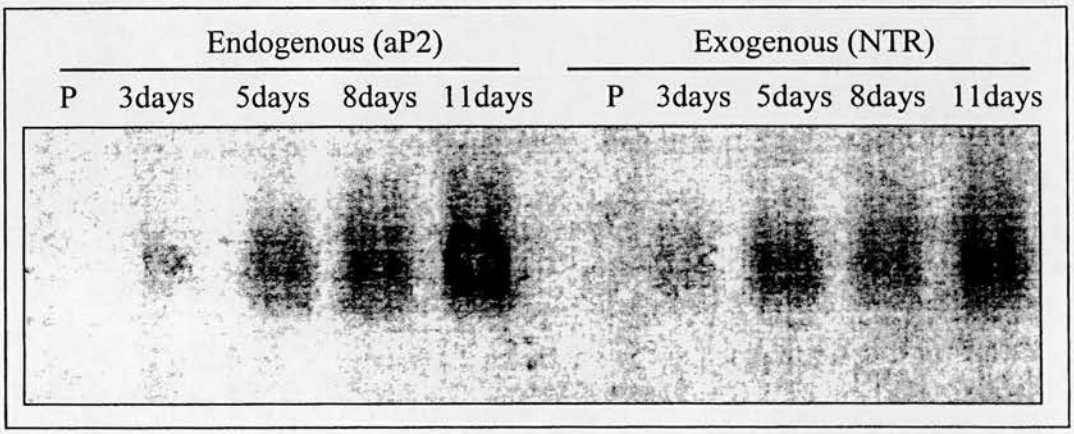



**Figure 3.10. Time course of appearance of both the endogenous aP2 and the exogenous aP2 driven NTR transcripts.**
Total RNA was collected at different time points as indicated in the figure and a Northern blot hybridised with probes to detect the endogenous (aP2) and the exogenous (NTR) genes.
P: Preadipocytes (before differentiation).
3,5,etc: Correspond to days after induction of the cells to differentiate.

## 3.6 NITROREDUCTASE-CB1954 MEDIATED ABLATION *IN VITRO.*

A pilot experiment was carried out where confluent dishes of cells transfected with the aP2-NTR plasmid (clone 10) and the parental cell line (3T3L1) were induced to differentiate in the presence of the prodrug CB1954. Cytotoxicity was observed in cells transfected with the *ntr* gene after 4 days of induction to differentiate in the presence of 100 μM of CB1954 but not in the parental cell line (Figure 3.11 B and E, respectively). The effect was seen as patches or clear areas on the dish where cells have disappeared (Figure 3.11 E). The effects seen in these cells are in agreement with the time course of activation of the aP2 promoter, which normally occurs on day 3 of the differentiation protocol (see Figure 3.1). This indicates that as soon as the promoter switches on, CB1954 is activated to the cytotoxic derivatives. The prodrug also caused gross changes in morphology of the cells, including enlargement of nuclei and multiple nuclei (Figure 3.11 F). These changes agree with some of the characteristic observed in apoptotic cells although apoptosis was not studied in this particular experiment. A similar effect was observed previously by Drabek *et al.* (1997) working with mouse L cells.

Unlike the cells transfected with *ntr* gene, parental cell lines (3T3L1 cells) are not affected when they differentiate in medium containing CB1954, indicating that NTR/CB1954 system is capable of mediating ablation exclusively in NTR expressing cells. Furthermore, transfected cells undergoing differentiation in medium without CB1954 differentiated normally and clusters of adipocytes could be observed as expected by day 4-5, indicating that the effect is due to the prodrug and not due to the insertion site of the transgene.

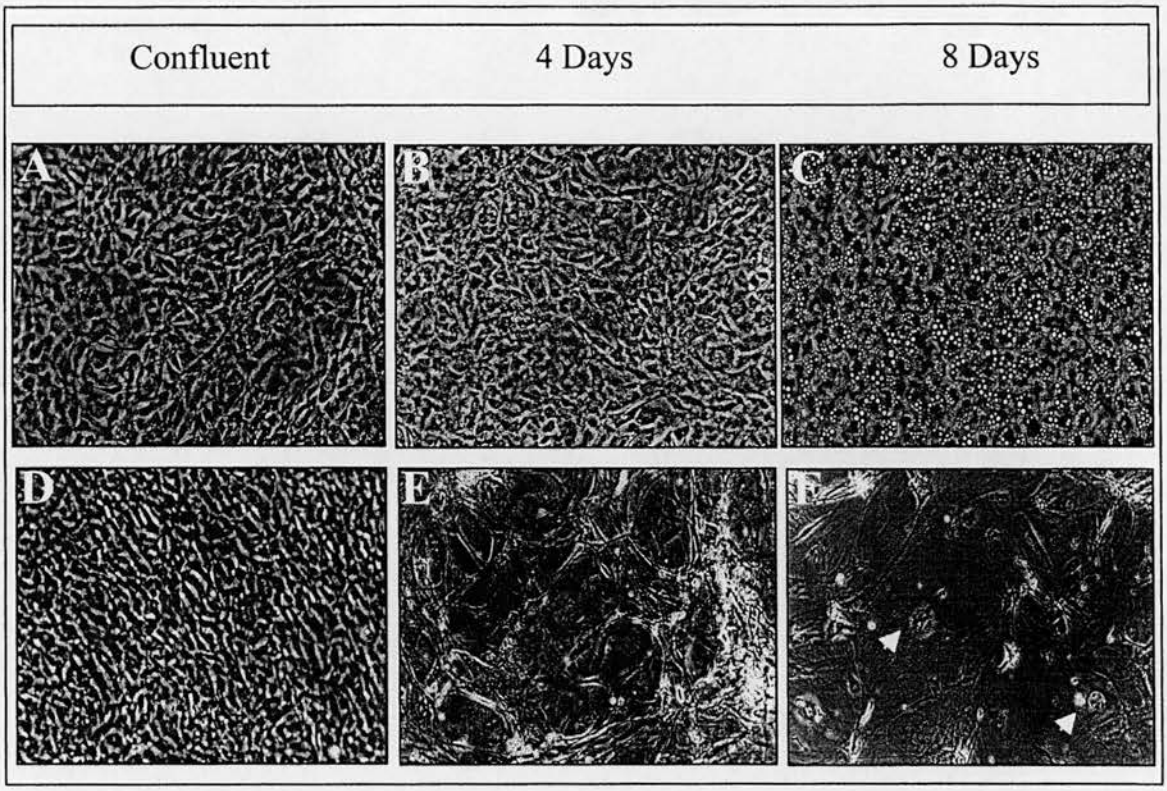



**Figure 3.11 Differentiation of preadipocytes in medium containing 100 μM CB1954**. Phase-contrast photomicrograph of parental 3T3L1 cells (A-C) and transfected cells (D-F) undergoing differentiation in the presence of 100 μM of the prodrug CB1954. Panels A and D show the cells at confluence stage when the hormones and the prodrug were added to the medium. Panels B and E correspond to the same cells after 4 days of the induction to differentiate and panels C and F after 8 days. Note how the cells have disappeared from the dish and remaining cells failed to differentiate (E and F) only in transfected cells (clone 10).
Arrows: Enlargement of nuclei and multiple nuclei are shown in cells undergoing apoptosis. Magnification: 100X.

## 3.7 DOES THE NITROREDUCTASE-CB1954 SYSTEM KILL ADIPOCYTES *IN VITRO*?

The main purpose of the work *in vitro* was to demonstrate the feasibility of the NTR/CB1954 system to kill adipocytes. It was thought that this could give proof of principle regarding the behaviour of the system in an animal model in terms of the expression of the bacteria enzyme (NTR) in adipocytes and the susceptibility of these cells to different concentration of the prodrug. In the previous section it was shown that the system could kill transfected cells once induced to differentiate in the presence of the prodrug. However, a different situation could have been expected from fully differentiated adipocytes due to the possibility of these cells being intrinsically more resistant to CB1954.

In order to demonstrate this, experiments were carried out this time applying the prodrug CB1954 once differentiation had proceeded to term, typically after 10 days of the induction with hormones. Differentiated cells expressing nitroreductase and parental cell lines were tested for their ability to survive after treatment with the prodrug at various concentrations. It was found that adipocytes are indeed susceptible to the killing by the prodrug CB1954 while parental cell lines were not affected. Both cells were grown in medium with the prodrug for 12 days and the number of cells were counted with an hematocytometer at different time points (days 0, 3, 5, 7, and 12). Transfected cells expressing NTR (clone 10) begun to die soon after 3 days of treatment and by 12 days around 80% of the cells had disappeared from the dish, leaving a few remaining cells that did not differentiate and presumably are not expressing the nitroreductase (see Figure 3.12). By contrast, parental cell lines were not affected by the prodrug and the cell number remained relatively constant, despite a slight reduction as a natural process of cellular death in culture (same effect was seen in 3T3L1 grown with and without the prodrug in the medium (see Figure 3.13 B).

The first signs of toxicity corresponded to a change in the phenotype of the cells (cell shrinkage) and membrane blebbing which is evident under these conditions after 3 days, concomitant with the cell detachment. By day 6 many cells have disappeared from the dish and this effect of the prodrug is irreversible after 4 days of treatment, since returning the cells to normal culture conditions did not lead

to their recovery. Optimal cytotoxicity was found with 200 μM leading to a 100% killing of the nitroreductase expressing cells with no apparent effect on the control cells (Figure 3.13 B). Here, the time required for maximum toxicity was half of the time to kill similar number of cells with 100 μM of the prodrug. This suggests that at lower doses of CB1954 the cells are still capable of repairing the DNA adducts formed as result of the bioactivation of CB1954 but above a certain concentration they are no longer capable of doing so. This threshold of toxicity will have critical implications for the survival of the cells as above certain concentration of the prodrug the cells will trigger a self-destructive response known as apoptosis.

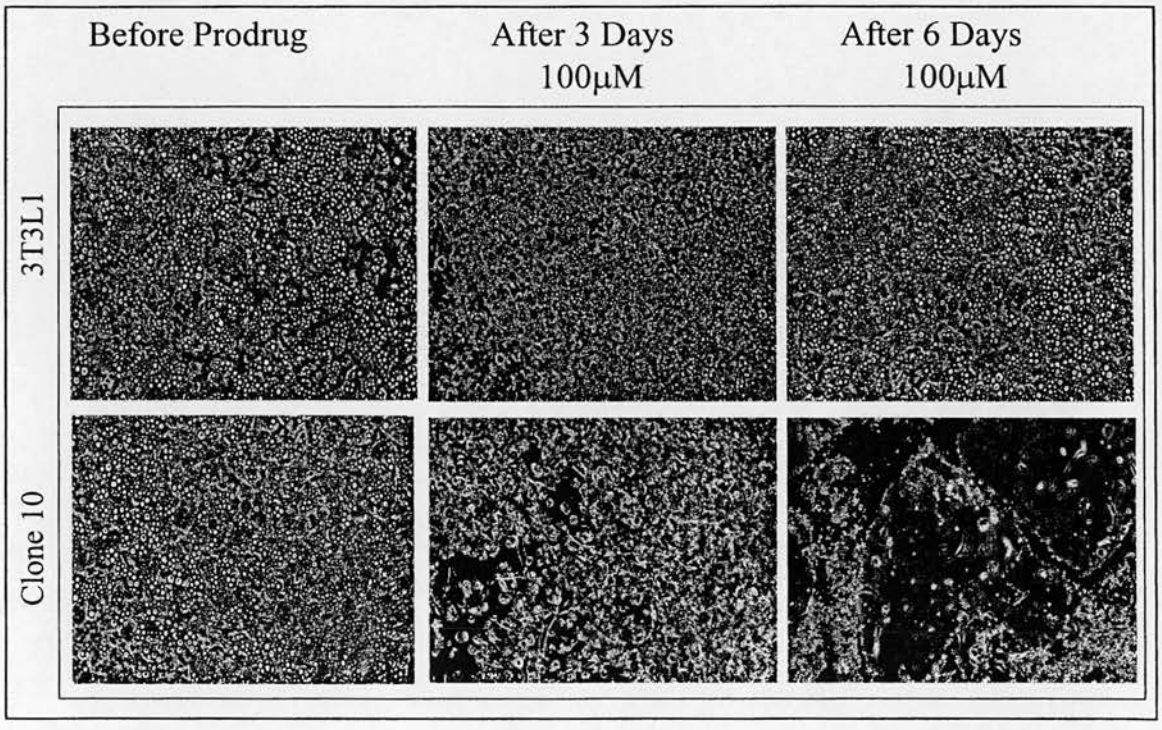
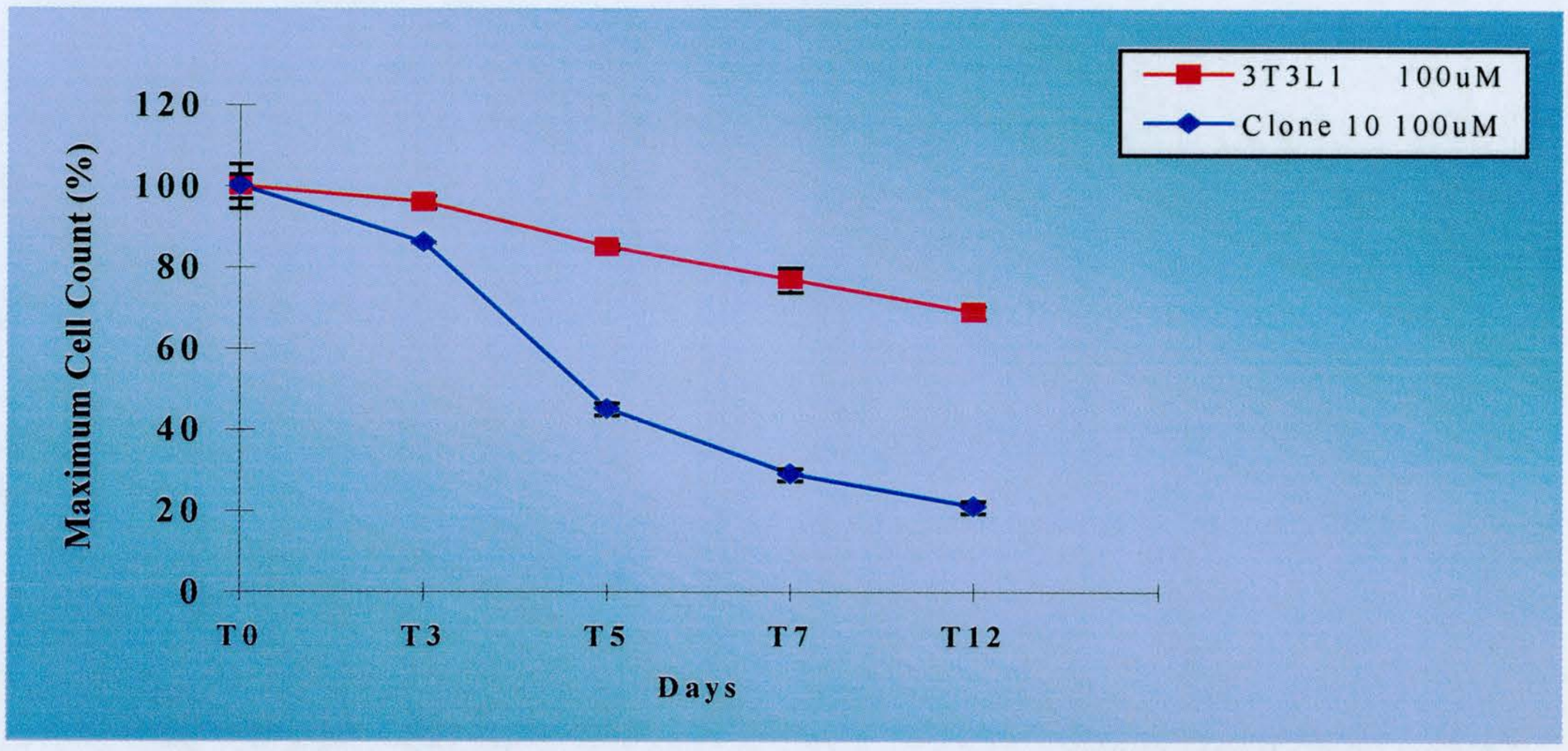



**Figure 3.12 Effect of CB1954 in adipocytes stably expressing NTR (clone 10) and the parental 3T3L1 cells.**
Phase-contrast photomicrograph to show that CB1954 treatment does not produce any toxic effect in parental 3T3L1 cells, however one of the clones (clone 10) stably expressing NTR show signs of toxicity after 3 days of treatment and after 6 days most of the cells have disappeared from the dish (see also Figure 3.13 A and B for quantitative analysis). Magnification 100X except middle panel 40X.

## EFFECT OF DIFFERENT DOSES OF CB1954 ON THE SURVIVING RATE OF THE ADIPOCYTES *IN VITRO*.

A) 100μM




B) 200μM

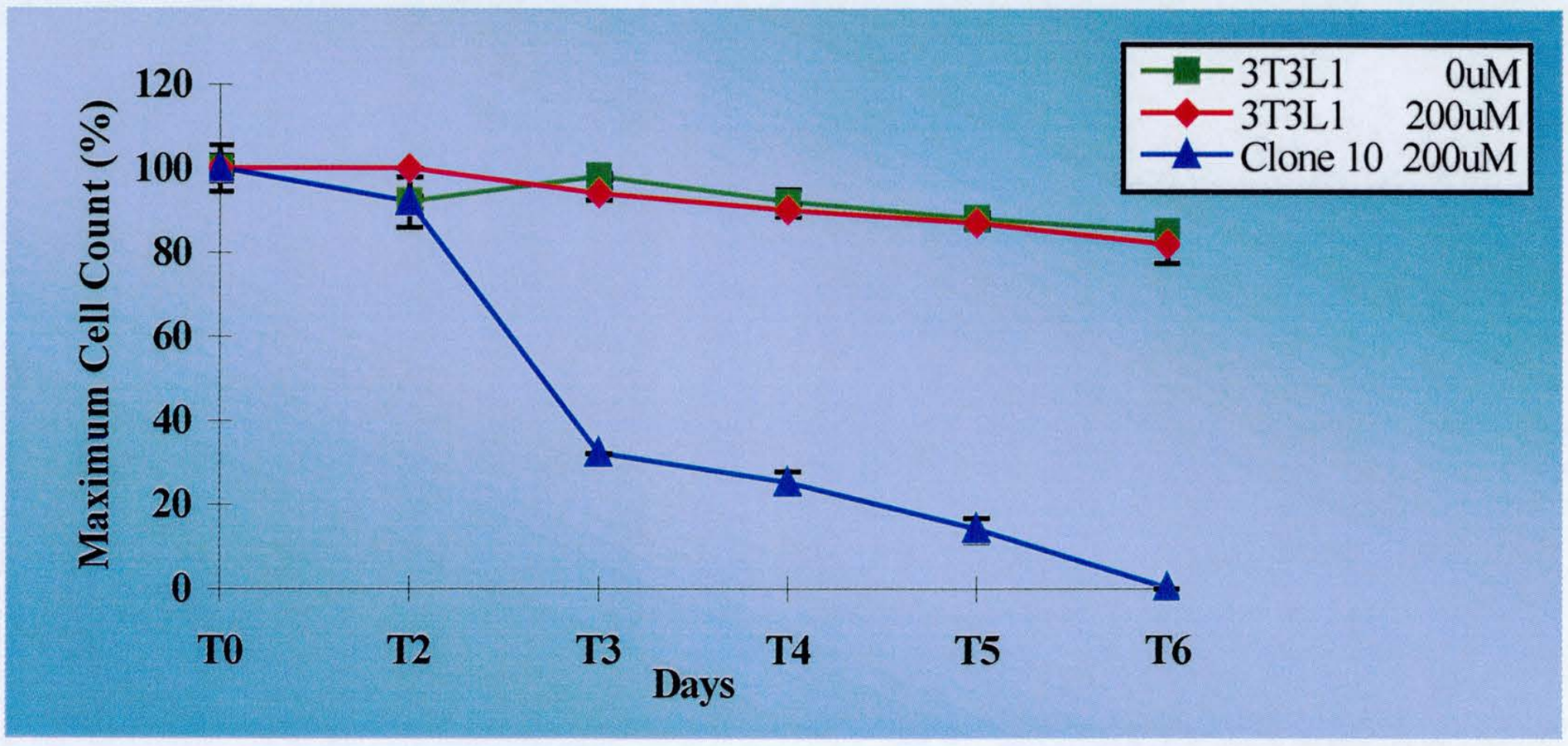



**Figure 3.13 Quantitative effect of CB1954 in the survival rate of adipocytes expressing NTR.** Differentiated parental cells (3T3L1) and one of the clones expressing NTR (clone 10) were incubated in medium containing the prodrug CB1954 at a final concentration of 100 and 200μM (panels A and B respectively). The number of cells was counted at different time points, as indicated in the graph. Each point represents the mean ± SD of duplicates.

The results showed above demonstrate the ability of the NTR/CB1954 system to kill non-dividing fully differentiated cells thus confirming that this system is a convenient way to eliminate terminal differentiated cells and might well work to eliminate other such as neurons and non-dividing neoplastic cells, a not infrequent component of any solid tumour. This is in contrast to the *tk*/GCV system, which requires cells to be in S phase for cytotoxicity. Thus, NTR/CB1954 system can prove a better alternative for the generation of transgenic animal models lacking of a specific cell type.

## 3.8 INCREASED EXPRESSION LEVEL LEADS TO A GREATER CYTOTOXICITY.

Section 3.4 characterised the expression of NTR transgene by Northern Blot and RT-PCR in different clones of 3T3L1 cells stably expressing NTR upon differentiation. Different levels of expression were observed (Figures 3.9 A and B) with some of clones expressing high levels (clone A and 10), while others at very low only detectable by the more sensitive RT-PCR assay (clone 3). Thus, it was decided to test the effect that CB1954 would have in the survival of these cells expressing different levels of NTR.

Increasing levels of enzyme expression in cell populations were found to correlate with their sensitivity to CB1954 (Figure 3.14). In this way, clones of cells expressing lower levels of NTR were more resistant to the killing by the prodrug when grown in similar conditions with clones expressing higher levels. These results suggest that those clones with the highest levels of NTR expression are more sensitive to the killing by the prodrug CB1954 because of the higher concentration of the *E.coli* nitroreductase enzyme available to metabolise the prodrug.

## DEGREE OF CELL KILLING CORRELATES WITH LEVELS OF NTR EXPRESSION.

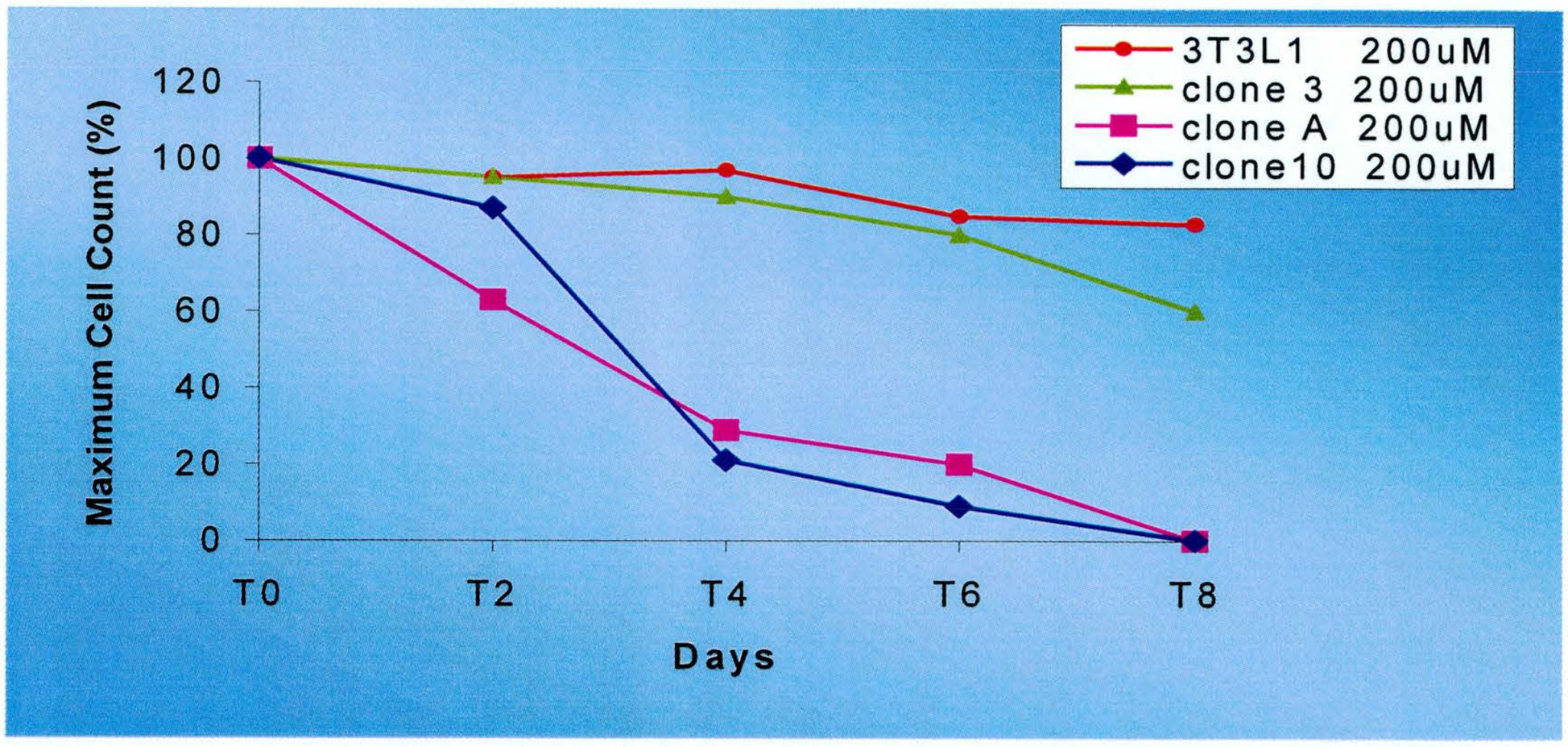



**Figure 3.14** Clones of cells 3T3L1 expressing different levels of the NTR enzyme were grown in medium containing 200μM of CB1954 and the cell number determined with an hematocytometer at different time points. Values correspond to single determinations.

### 3.9 DOSE-DEPENDENCE OF *IN VITRO* CYTOTOXICITY OF THE NTR/CB1954 SYSTEM.

The therapeutic index (toxicity in parental cells versus toxicity in transfected cells) of this suicide gene/prodrug system was determined by analysing the $LD_{50}$ or the concentration of prodrug required to kill 50% of the cells. As shown in Figure 3.15, the $LD_{50}$ in the parental cells was 2.500 μM and in NTR expressing cells (clone 10) was 150 μM resulting in the therapeutic index of 17. A vast increase in its cytotoxicity of up to 100.000 fold was originally found *in vitro* in the extremely sensitive Walker cells compared to more resistant Chinese hamster V79 cells (Roberts *et al.* 1986). Despite this high potency, CB1954 was not effective in a range of animal and human tumours (Workman *et al.* 1986). This lack of antitumour response in humans was due to species differences in CB1954 reduction, catalysed by the nitroreductase enzyme DT-diaphorase (Knox *et al.* 1993). Nevertheless, in several human and murine cells, following delivery of the *E.coli* NTR gene, which

can also catalyse CB1954 reduction, differential toxicity between cells expressing the NTR gene and non-expressing control cells has reached 10-100 fold (Bridgewater *et al.* 1995; Clark *et al.* 1997; Drabek *et al.* 1997), therefore making this system as remarkable as that observed with *tk*/GCV. Furthermore, from the experiments already described in section 3.8 is clear that the cytotoxicity of nitroreductase–expressing cells is directly correlated to the levels of enzyme produced from these cells. Thus, it is possible that this therapeutic index could be even greater if a clone expressing higher levels of NTR is selected for this analysis.

Most of the experiments carried out to date with NTR/CB1954 have used actively dividing cells rather than fully differentiated. This therapeutic index observed in differentiated adipocytes thus confirms the feasibility of this system to be used in a transgenic animal model to kill fully differentiated cells.

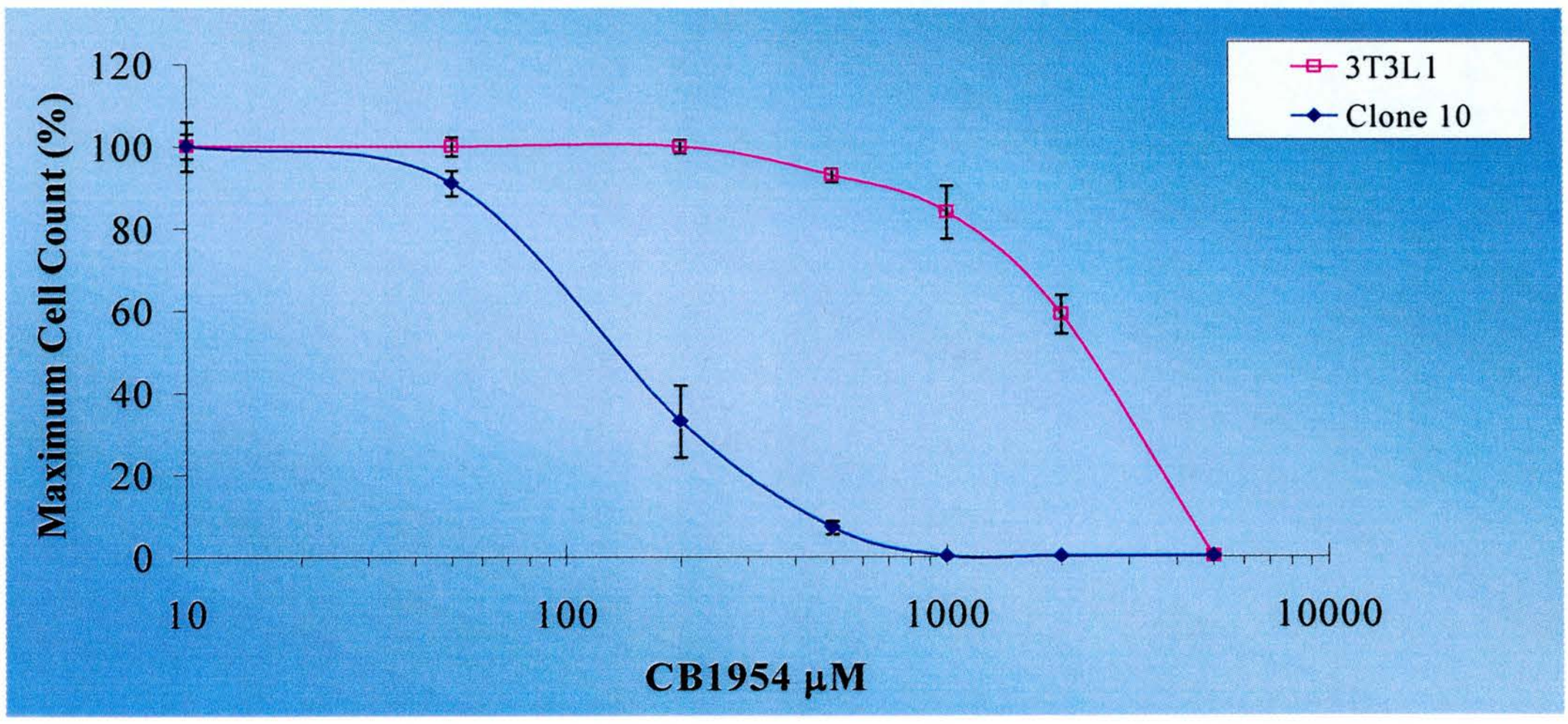



**Figure 3.15 Dose-dependence of *in vitro* cytotoxicity of the NTR/CB1954 system.**
3T3L1 cells stably expressing NTR (clone 10) and parental cell lines (3T3L1) were incubated with increasing concentrations of the prodrug CB1954 and the number of surviving cells counted after 72 hours of incubation. Each point represents the mean ± SD of duplicates. X axis is logarithmic. Open and closed symbols represent the data obtained with parental and transfected cells, respectively.

## 3.10 THE MECHANISM OF NTR/CB1954 MEDIATED ABLATION IS APOPTOSIS.

Apoptosis or programmed cell death represents a physiological form of cell death that occurs during development and in the mature animal. Cell culture provides many advantages to studying apoptosis, including the availability of large quantities of homogeneous cells, the ability to examine the morphological, biochemical and genetic properties of cells undergoing apoptosis throughout the course of experiments, and the ability to precisely regulate the cellular environment.

This section describes two approaches that were used to measure apoptotic cell death of adipocytes undergoing NTR/CB1954 mediated ablation. The first method used was a commercial Tunel assay (Figure 3.16) which takes advantage of the biochemical hallmark of apoptosis, namely internucleosomal DNA fragmentation, to chemically label and visualise apoptotic nuclei. This method (described in Chapter 2) utilises the activity of terminal deoxynucletidyl transferase (TdT) enzyme to incorporate biotinylated dUTP onto 3' ends of fragmented DNA. The incorporated biotinylated-dUTP is commonly visualised after incubation of the labeled cells with avidin-biotin-conjugated horseradish peroxidase followed by staining with 3,3'-diaminobenzidine tetrahydrocholoride (DAB). Figure 3.16 shows the result of the Tunel assay carried out on adipocytes after treatment with 200μM of CB1954 for 4 days. Note the specific brown colour staining indicative of apoptosis mediated DNA strand breaks only in NTR expressing cells treated with the prodrug, thus confirming apoptosis of these cells.

A second method (DNA laddering) was also used to confirm the results obtained with the Tunel assay. Cleavage of genomic DNA into oligonucleosome-length fragments by endogenous nucleases is the biochemical hallmark of apoptosis in nearly all systems examined to date (Bortner *et al.* 1995). As can be seen from the Figure 3.17 a characteristic DNA laddering could only be observed in NTR-expressing cells that had been treated with the prodrug (lane 9 in Figure 3.17).

# MECHANISM OF ADIPOCYTE KILLING IS APOPTOSIS

| 3T3L1 100μM CB1954 | Clone 10 100μM CB1954 |
|---|---|

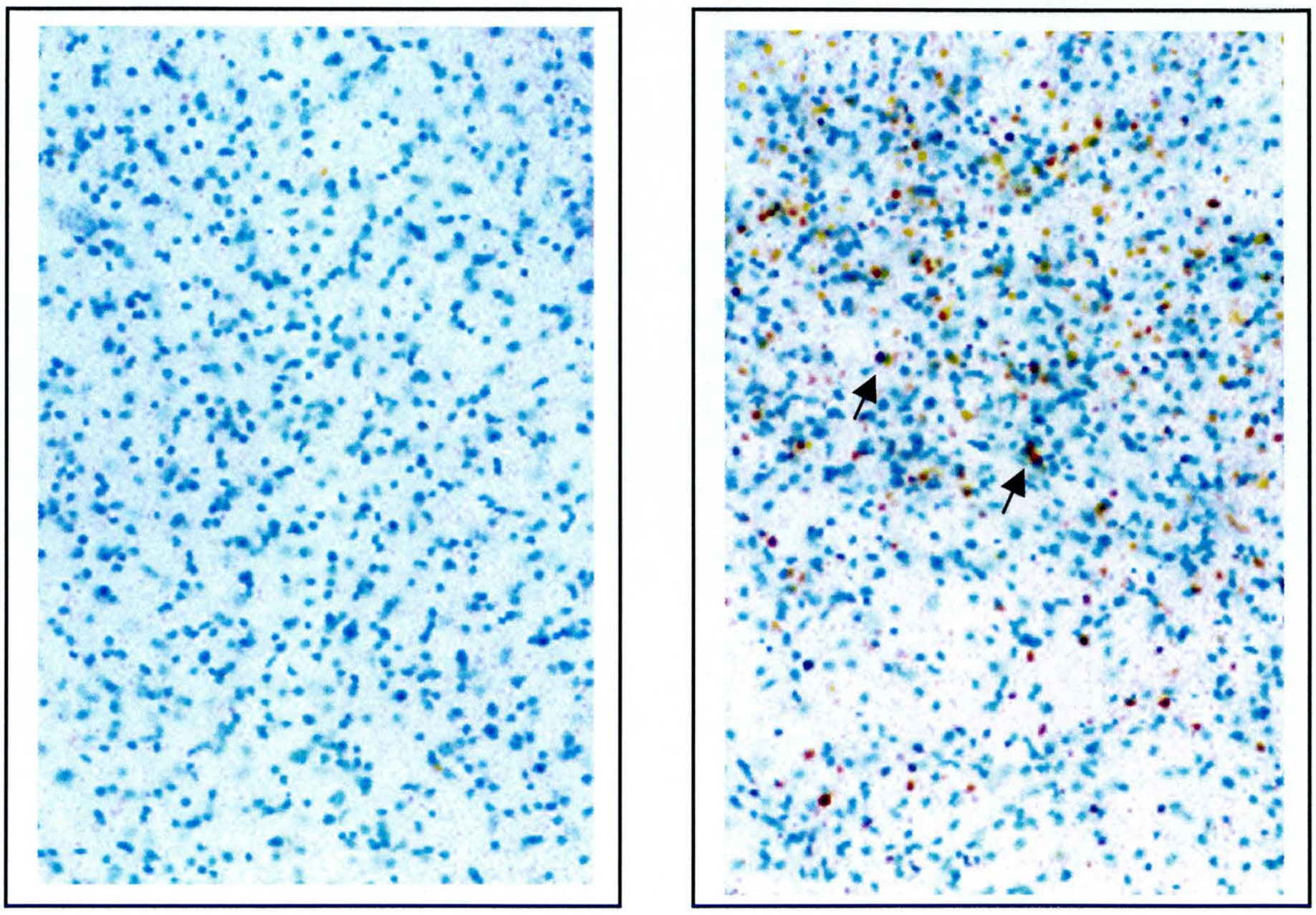


**Figure 3.16 Tunel Assay.**
The Figure shows the result of the Tunel assay carried out in cells expressing the nitroreductase (clone 10) and the parental cell lines (3T3L1). Cells were treated with 100μM of CB1954 for 4 days. Note the characteristic brown colour (arrows) indicative of apoptotic nuclei only seen in those cells expressing NTR and treated with CB1954 (Clone 10). Magnification 200X.

Although a number of protocols were used to detect this DNA laddering, the one that gave the best results is the described by Sellins and Cohen, (1987) as shown in Figure 3.17. This protocol (described in detail in Chapter 2) contains a preliminary centrifugation step that allows the recovery of fragmented DNA contained in the supernatant fraction. This DNA is later precipitated with isopropanol in the presence of salts. The pellets obtained in the first centrifugation contain the remaining genomic DNA, which is later digested with buffer and proteinase K and finally precipitated. These two fractions, genomic and supernatant DNA respectively, were then loaded onto 1.5% agarose gels.

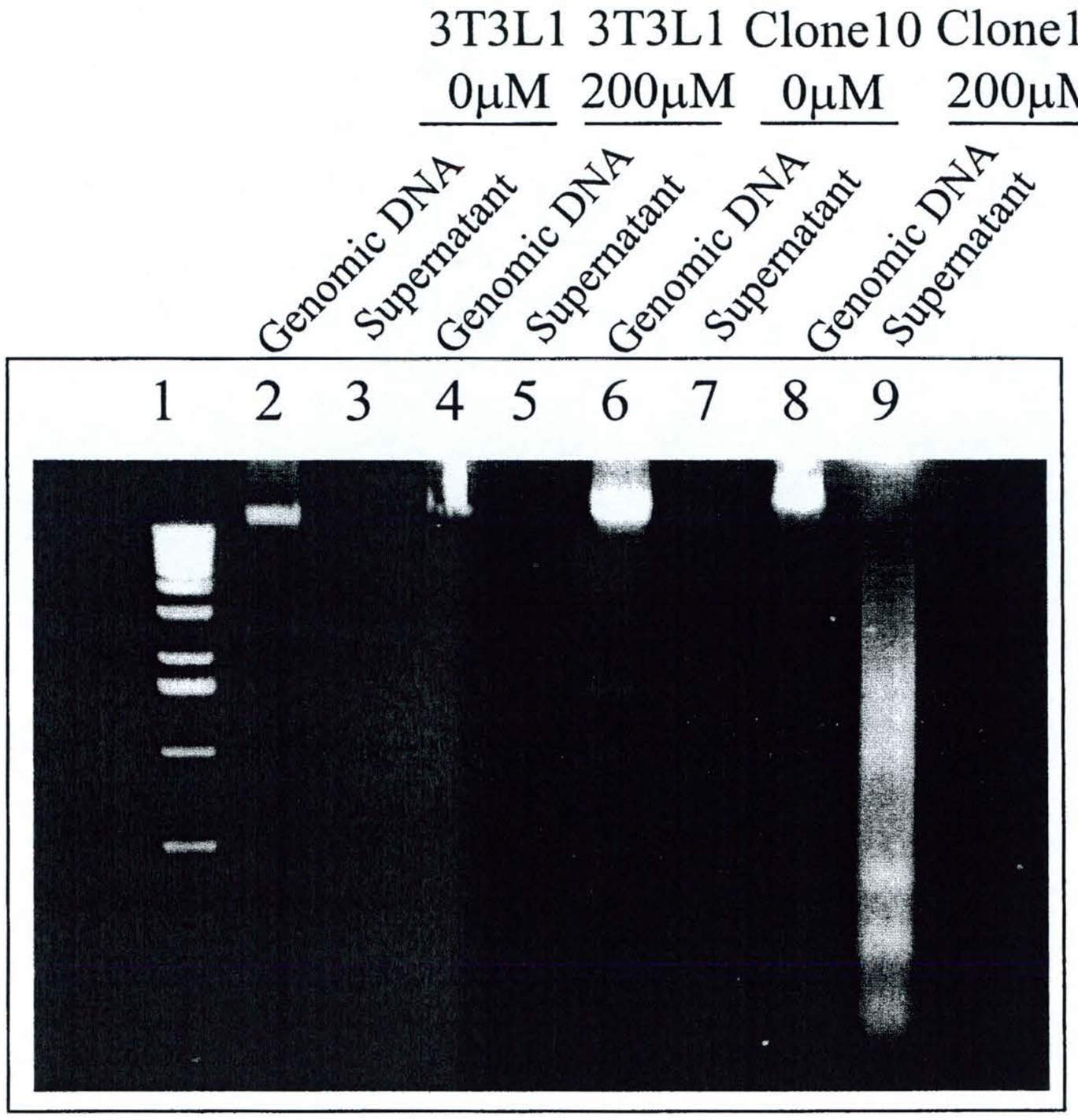



**Figure 3.17 DNA fragmentation assays.**
Lane 1 1kb marker. Lanes 2 and 3 correspond to genomic and supernatant DNAs of the parental cell line (3T3L1) without treatment. Lanes 4 and 5 correspond to genomic and supernatant DNA of the parental cell line (3T3L1) treated with 200μM of CB1954. Lanes 6 and 7 correspond to genomic and supernatant DNA of the nitroreductase expressing cells (clone 10) without treatment. Lanes 8 and 9 correspond to genomic and supernatant DNA of the nitroreductase expressing cells (clone 10) treated with 200μM of CB1954. Note the effect (DNA laddering) seen only in those cells expressing NTR that were treated with the prodrug.

The results shown above confirmed that the mechanism of ablation is mediated by apoptosis, which is in agreement with the proposed cytotoxic role of the DNA adducts formed after activation of the prodrug (Friedlos *et al.* 1992).

## 3.11 NTR/CB1954 HAS A BYSTANDER EFFECT THAT IS MEDIATED THROUGH A METABOLITE RELEASED INTO THE MEDIUM.

The ability of the NTR/CB1954 system to kill NTR non-expressing cells was assayed by mixing the parental cell lines (3T3L1) with different proportions of one of the clones stably expressing the NTR enzyme (clone 10). Cells were mixed at the following proportions: (parental 3T3L1:Clone 10) 100%:0%, 50%:50%, 30%:70%, 10%:90%, and 0%;100%. Upon differentiation of the cells to adipocytes, 200μM of the CB1954 was added and the cells incubated for 96 hours after which time, surviving cell numbers were counted with the help of an hematocytometer. Figure 3.18 confirmed that killing by this bystander effect is also observed in adipocytes expressing the NTR enzyme. 50% of NTR expressing cells were enough to kill over 70% of the cell population under the conditions used in this experiment. It is noteworthy that this number may not reflect the real potential of the bystander effect of these cells as 100% conversion to the adipocyte estate it is unlikely to be obtained in culture conditions (3T3L1 can only differentiate with a 90-95% efficiency). This might imply that the 50% of NTR expressing cells used might only have contained a 40-45% of cells expressing the NTR.

Another situation that might underestimate this potential bystander effect is the possibility of variegated transgene expression in these cells expressing the NTR, a not uncommon phenomenon in culture systems. Some effort was devoted to assess for the possibility of variegated transgene expression in one of these clones stably expressing the NTR. Immunocytochemistry analysis with antiserum to NTR was used to estimate the proportion of cells that were expressing the NTR within the clone. Unfortunately, due to cross-reactivity of the antibody it was not possible to distinguish between expressing and not expressing cells since both showed immunostaining (data not shown). No further attempts were made to address this point.

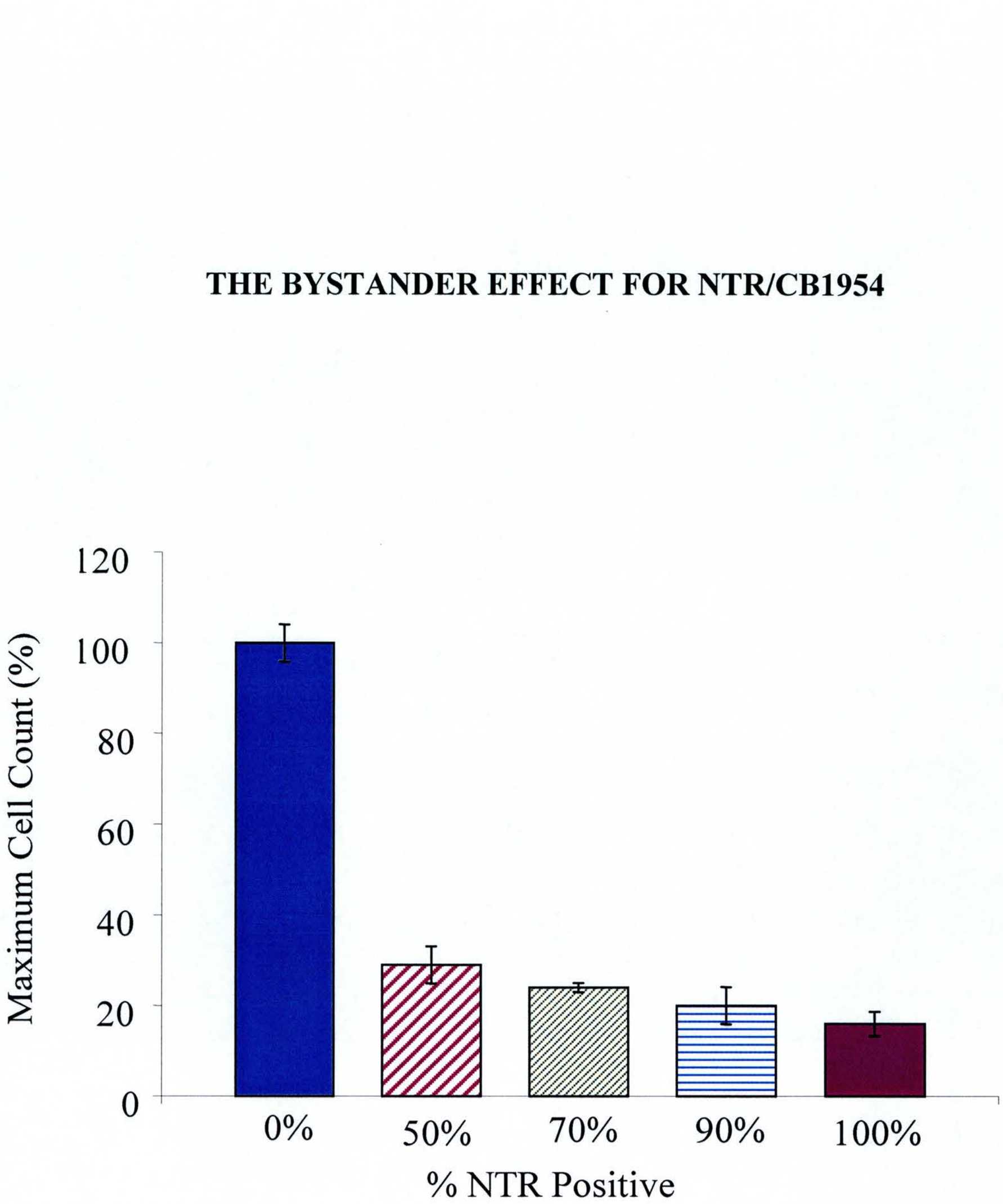



**Figure 3.18 In vitro bystander effect of the NTR/CB1954 system.** Parental 3T3L1 cells were mixed with various proportions of the 3T3L1 cell line stably expressing the NTR (clone 10) and exposed to 200μM of CB1954 for 96 hours. After the incubation period cell number were counted as described above. Each bar represents the mean ± SD of duplicates.

## 3.12 CYTOTOXICITY CAN BE TRANSFERRED IN THE CONDITIONED MEDIUM (CMED) OF 3T3L1 CELLS EXPRESSING NTR WHEN TREATED WITH CB1954.

NTR expressing and non-expressing (parental cell lines) were treated with media alone or media in the presence of 200μM of the prodrug for 96 hour. After this time CMED was collected from each of these dishes and transferred (after being filtered with 0.2μm filters) to parental 3T3L1 cells and left for 96 hours. When conditioned medium (CMED) from one of the clones expressing nitroreductase (clone 10) treated with CB1954 was transferred to 3T3L1 cells (Figures 3.19 and 3.20 D), cell survival was reduced to 50% when compared to CMED from clone 10 without CB1954, and CMED from parental cells with or without CB1954. This data confirmed the presence of a diffusable active metabolite of CB1954 that passed into the CMED and that was sufficiently stable to confer cytotoxicity following transfer.

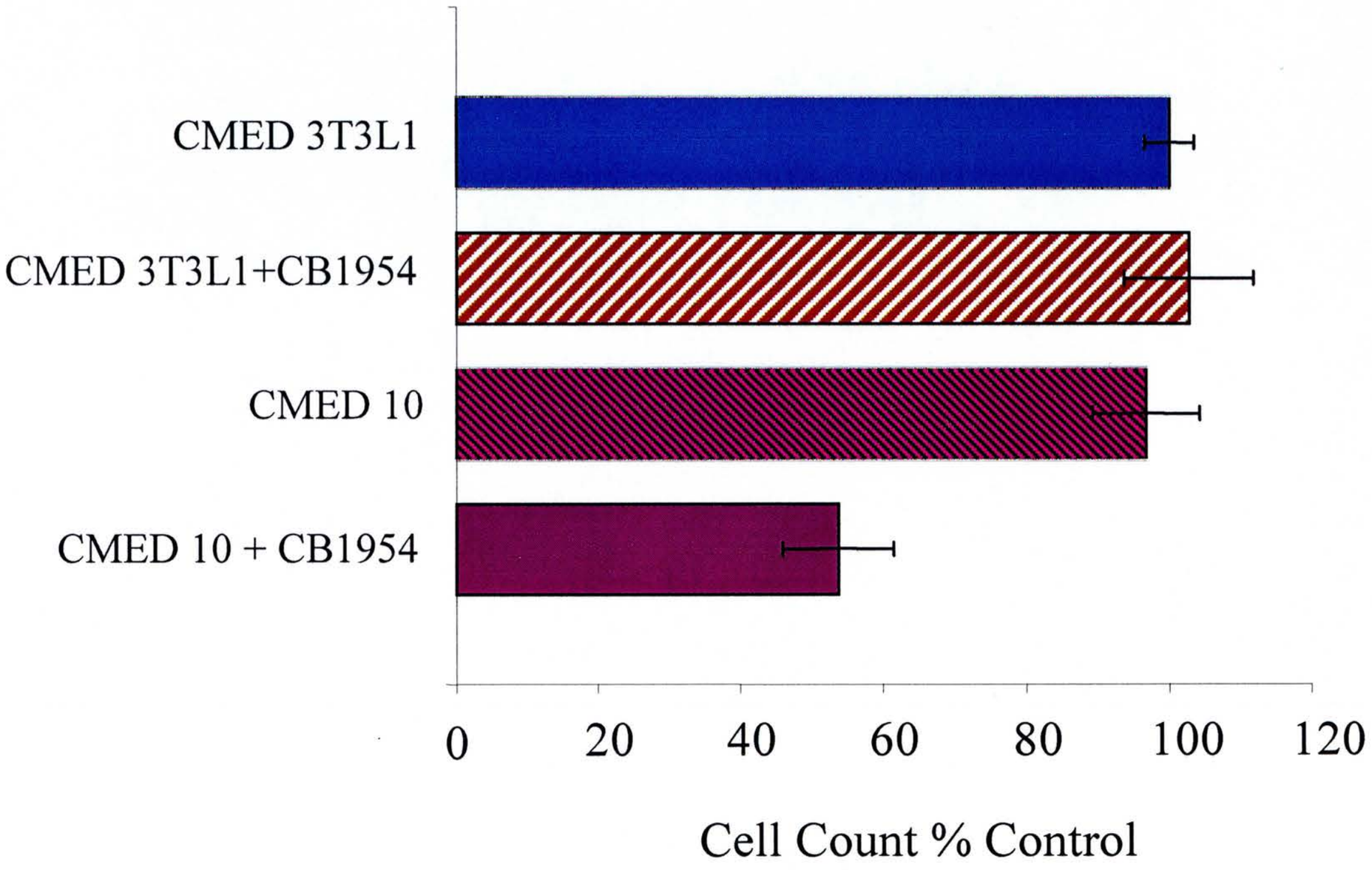



**Figure 3.19 Toxicity of CMED from NTR expressing cells.**
3T3L1 stably expressing NTR (clone 10) and parental cell lines (3T3L1) were treated with media alone or media in the presence of the prodrug. The CMED was transferred after being filtered to 3T3L1 cells and the number of surviving cells counted after 96 hours. The y axis shows the following conditions: 3T3L1 CMED alone, 3T3L1 CMED with prodrug, clone 10 CMED alone, clone 10 CMED with prodrug. Each bar represents the mean ± SD of triplicates.

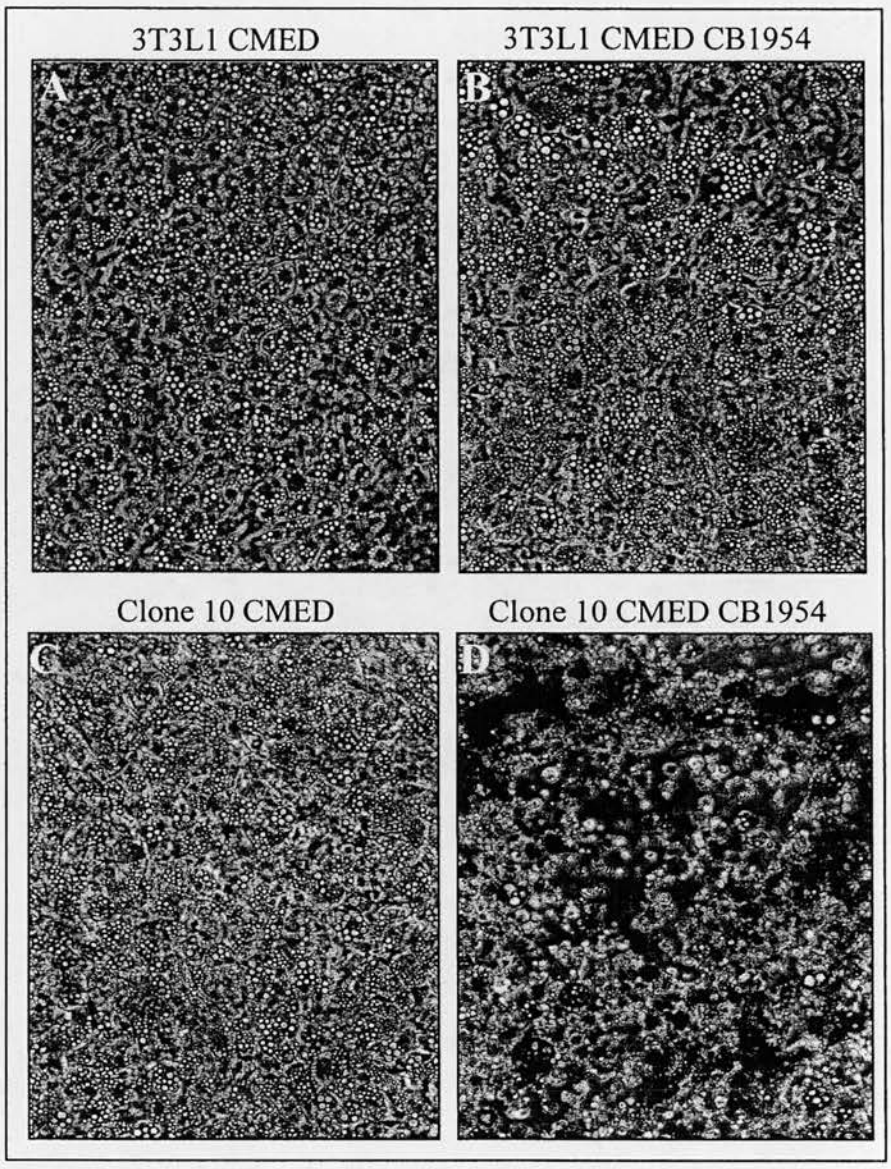



**Figure 3.20 Conditioned medium toxicity on parental 3T3L1 cells.**
Phase-contrast photomicrograph of the effects of supernatant derived from 3T3L1 either untreated or CB1954-treated (A and B) or NTR expressing (clone 10) untreated or CB1954-treated, on the wild-type 3T3L1 cells.
A, B and C show the 3T3L1 cells growing well in CMED derived from parental 3T3L1 cells untreated (A) and treated with CB1954 (B) and CMED derived from NTR expressing cells untreated (C). In contrast, when CMED was derived from NTR expressing cells that had been treated with CB1954 a considerable cell death could be observed. Magnification 100X.

These results indicate the presence of a toxic metabolite of CB1954 that is relatively stable and more importantly that can freely cross the cellular membrane into the culture medium to mediate a bystander effect, in agreement with previous observations of Bridgewater *et al.* (1995). This mechanism of bystander effect differs from the classical mechanism of intercellular gap junctional communication observed with *tk*/GCV system. Nevertheless, due to the proximity of one cell each other, as it is the case in confluent adipocytes, it can not be rule out the possibility of other mechanisms acting synergistically to enhance this bystander effect.

A number of studies attempting gene delivery to tumours *in vivo* have shown that a proportion of cells remains unmodified. Thus, a desirable feature of any enzyme/prodrug combination for use in GDEPT is that activation and bystander effects of the modified cells could also kill adjacent unmodified cells. Here it was further confirmed that a bystander effect, as observed with *tk*/GCV, is also present with NTR/CB1954 system. This bystander effect may have important implications in the killing of surrounding non-infected tumour cells in the case of gene therapy or non-expressing cells when used to ablate specific cells in transgenic animal models (Knox *et al.* 1988; Clark *et al.* 1997; Drabek *et al.* 1997).

## 3.13 CONCLUSION.

3T3L1 cells were successfully transfected with the *E.coli ntr* gene driven by the adipocyte specific promoter aP2. Activation of the transgene by the promoter sequences used indicates that *E.coli* NTR is not detrimental for the survival of the cells and more importantly for an appropriate differentiation of these cells into adipocytes. The aP2 promoter sequences used contain the necessary regulatory elements to drive the expression of exogenous genes into adipocytes *in vitro* in a temporal developmental manner similar to the endogenous gene.

CB1954 is converted by nitroreduction from a monofunctional to a difunctional alkylating agent capable of reacting with DNA to produce interstrand crosslinks. The results described above showed that differentiated eukaryotic cells

are able to express the *E.coli* nitroreductase gene and judging for the end result (cell death), are capable of reducing this substrate, inducing DNA interstrand crosslink formation leading to cell death.

The degree of cytotoxicity correlates with the level of nitroreductase activity in these cells, presumably due to a more efficient conversion of the prodrug to the active metabolites, in agreement with previous work in murine fibroblast and other human tumour cell lines (Bridgewater *et al.* 1995; Green *et al.* 1997; McNeish *et al.* 1998). In the same way, low concentration of the prodrug rendered these cells more resistant to the killing, requiring longer time exposure to the prodrug to achieve a similar toxic effect. One possible explanation for this effect is that at lower concentrations the cells are still capable of repairing the DNA adducts formed as a result of the bioactivation of the prodrug and over certain concentration they are no longer capable of doing so. As a consequence of these lesions it has been suggested that cells react triggering a self destructive response known as apoptosis. Here, it was confirmed that apoptosis is involved in the mechanism of cell killing of adipocytes. Both morphological and cellular data are consistent with this observation. TdT-mediated dUTP nick end labeling (Tunel) showed specific staining indicative of apoptosis-mediated DNA strand breaks in cells expressing NTR treated with the prodrug. Furthermore, DNA fragmentation and morphological analysis confirmed the apoptosis-mediated cell death.

In trials of gene therapy using HSV-tk, it has been shown that tumours regress even when only a fraction of the tumour mass contains the viral gene (Freeman *et al.* 1993). Furthermore, a few cells transfected with the HSV-tk gene without retroviral infectious capabilities were also able to cause tumour regression (Ram *et al.* 1993). This implies that in the presence of ganciclovir, cells that produce HSV-tk are able to cause the death of neighbouring cells that do not themselves produce the enzyme. This phenomenon has been termed the "bystander effect" (Kolberg R. 1992). A similar bystander effect has been previously observed with the NTR/CB1954 system by some authors (Knox *et al.* 1988), although the same effect could not be observed by others (Clark *et al.* 1997). Here, it was further confirmed

the ability of a minority of nitroreductase expressing cells to cause prodrug-dependent death of surrounding nitroreductase non-expressing cells. An efficient bystander effect is widely acknowledged to be an essential feature of enzyme/prodrug systems for potential applications in GDEPT, or VDEPT as gene transfer to 100% of tumour cells *in vivo* is unlikely to be achieved. In the same way, this bystander effect is especially useful with transgenes affected of variegated expression; a not uncommon problem in transgenesis thus enabling the killing of surrounding silenced cells.

These studies allow to conclude the feasibility of the NTR/CB1954 system to kill adipocytes *in vitro*. However, these studies do not guarantee that the system would work in an animal model where the levels of transgene expression are more likely to be affected by environmental factors and external cues. CB1954 can also have a major role for the success of the experiments *in vivo* since it must be administered to such concentration that can reach the organ target to levels high enough to kill the NTR expressing cells. Therefore, in order to confirm that the system works *in vivo* and specifically in the target tissues, it is necessary to create a mouse model expressing NTR in the adipose tissue and assess the susceptibility of these cells to the prodrug as it will be described next.

# 4. TRANSGENIC MICE HARBOURING *E.COLI* NITROREDUCTASE GENE DRIVEN BY THE aP2 PROMOTER ENHANCER: GENERATION AND ANALYSIS.

## 4.1 BACKGROUND.

Transgenic animal technology has become one of the most fascinating technologies developed in the last two decades. The introduction of foreign genes termed "transgenes" into the germline of animals is achieved by microinjection of multiple copies of the transgene into the pronucleus of a fertilised one-cell embryo. The transgene randomly integrates into the genome (usually at a single site) and the cleaved embryos (two-cell stage) are transferred to pseudopregnant females (females mated with vasectomised males). The live births can then be screened for the presence of the transgene either by PCR or Southern Blot analysis of DNA from tail biopsies. These transgenic animals exhibit unique phenotypes and these characteristics are inherited by their offspring in a Mendelian fashion. The mechanisms involved in the integration event are largely unknown but result in the insertion of different copy numbers of the transgene, usually in a direct head to tail array. In many cases the transgene is susceptible to "position effects", resulting in aberrant or different expression levels between lines carrying the same construct (Palmiter and Brinster, 1986).

One of the main drawbacks of this technique has been the low efficiency. Of the embryos that survive the injection procedure only ~10% produce offspring, of which ~20% are transgenic (Boyd and Samid 1993). Other problems associated with pronuclear injection are that neither the site of integration nor the number of copies of foreign DNA can be controlled precisely, which means that not infrequently some transgenes are silenced. In spite of the low efficiency, pronuclear injection is currently the most commonly used technique for the generation of transgenic animals and transgenic animals still provide unique opportunities to address

fundamental questions on development, gene regulation, cell biology, disease pathology and genetic engineering of animals to improve their health status.

This chapter describes the generation of transgenic mice harbouring the *E.coli* nitroreductase gene under the control of the adipocyte specific promoter aP2. The establishment of transgenic lines of mice and the analysis of transgene expression are also described.

## 4.2 TRANSGENE CONSTRUCT.

The nucleotide sequence of the *E.coli ntr* gene has been determined (Michael *et al.* 1994). The enzyme is a monomeric FMN-containing flavoprotein with a molecular mass of 24.000 Da and requires either NADH or NADPH as a cofactor. From comparison of partial aminoacid sequence the enzyme appears homologous to the classical nitroreductase of *Salmonella typhimurium* (Watanabe *et al.* 1990). The generation of the construct aP2-NTR has been described in section 3.2.1 in chapter 3. The same construct that was used to transfect cells in the previous section was used in the generation of transgenic mice as described next.

## 4.3 TRANSGENE MICROINJECTION.

Approximately 10 μg of the aP2-NTR plasmid was double digested with *Sal* I/*Not* I (Figure 4.1) and the transgene purified free of any plasmid sequences, which are thought to interfere with the stable integration of the transgene (Palmiter and Brinster, 1986). The concentration of the transgene DNA was determined by agarose gel analysis comparing with a standard of known concentration and aliquots of 60 ng/μl stock prepared in TE-buffer. The DNA was microinjected at a concentration of 1.5 ng/ml into pronuclear stage eggs from superovulated (C57Bl/6xCBA F1) females by Roberta Wallace.


**Figure 4.1 Map of the 6.95 kb aP2-NTR transgene.**
Probe 1 (679 bp) and probe 2 (850 bp) are indicated.

## 4.4 ANALYSIS OF THE FOUNDERS AND THE ESTABLISHMENT OF TRANSGENIC LINES OF MICE.

Transgenic founders were identified by PCR and Southern Blot analysis of mouse tail DNA. PCR analysis was carried out using a pair of primers that amplify a 471 bp fragment of the NTR gene. Figure 4.2 illustrates the result of a typical PCR reaction where the positive and negative mice are clearly seen. The control included in the PCR reaction corresponded to DNA from a transgenic and non-transgenic mouse (positive and negative controls, respectively), conditions of the PCR reaction were as described in chapter 2.

Southern Blot analysis was routinely used to confirm the results of the PCR reactions. Figure 4.3 is a Southern blot of all the founder mice that were positive by the PCR. 10 μg of mouse tail genomic DNA was digested with *EcoR* I, which cut once in the transgene, and Southern blot hybridised with probe 1 (Figure 4.1). This probe detects a 6.95 kb band corresponding to the transgene in a head to tail array. Thus, the presence of this fragment confirmed the integration of the transgene into the founder mice. The different intensities of the band indicate the variation in copy numbers of the transgene although a more accurately study of the copy number is shown later in this chapter. Out of 97 live births, 12 mice were positive by PCR and Southern blotting, an efficiency of 13%.

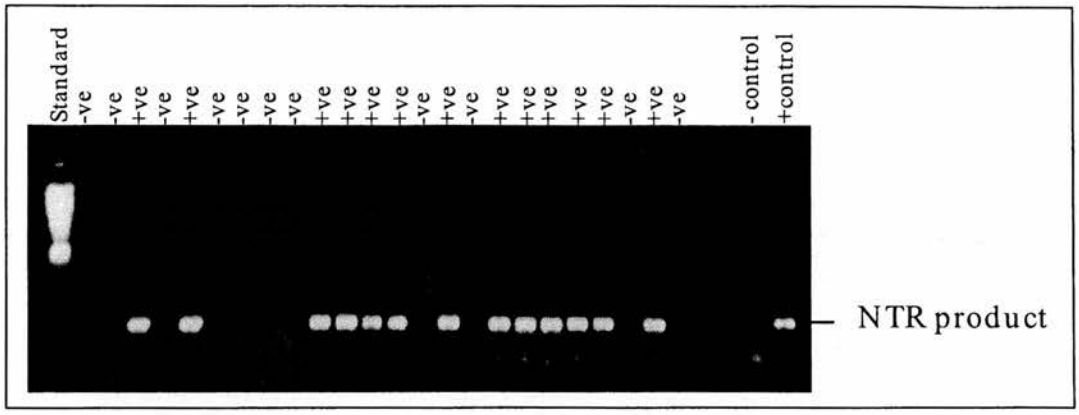



**Figure 4.2 Screening of genomic mouse tail DNA for transgenic mice carrying the transgene.**
Positive and negative control DNA samples are loaded on the right of the gel.

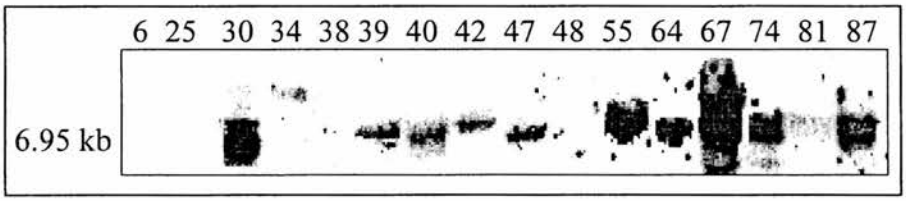



**Figure 4.3 Southern Blot of genomic tail DNA from different transgenic founders carrying the transgene.**
10 μg of genomic DNA was digested with *EcoR* I and Southern blot hybridised with probe 1 (Figure 4.1).

## 4.5 ANALYSIS OF THE TRANSGENE COPY NUMBER IN THE LINES OF MICE.

The copy number of the transgene in each line of mice was estimated by Southern blot analysis (Figure 4.4). Genomic DNA was prepared from liver tissue from 2 G1 sisters of each transgenic line. Copy number control was prepared by

adding different amounts of the transgene DNA (equivalent to 1, 5 and 25 copies) to a liver DNA sample known to be negative for the transgene. The DNA (10 μg) was digested with *Kpn*I, which cuts once in the transgene and the Southern blot hybridised with a 850 bp probe corresponding to the pA fragment (probe 2 in Figure 4.1). This probe detects a 6.95 kb band corresponding to the transgene in a head to tail array (Figure 4.5). The same Figure also shows other possible rearrangements. DNA loading was corrected by reprobing the blot with the murine cystic fibrosis transmembrane-conductance regulator (CFTR) probe, a single copy rodent gene (kindly provided by Dr. W. Cui). Figure 4.4 shows that the transgene is present in various copy numbers ranging from 1 (line 64) to over 25 (line 87). Please note that Figure 4.4 was overexposed to allow detection of the single copy control and the single copy signal from line 64. Also from the figure note that line 30-2 is overloaded when compared with its sister 30-1 (compare with loading control probe).

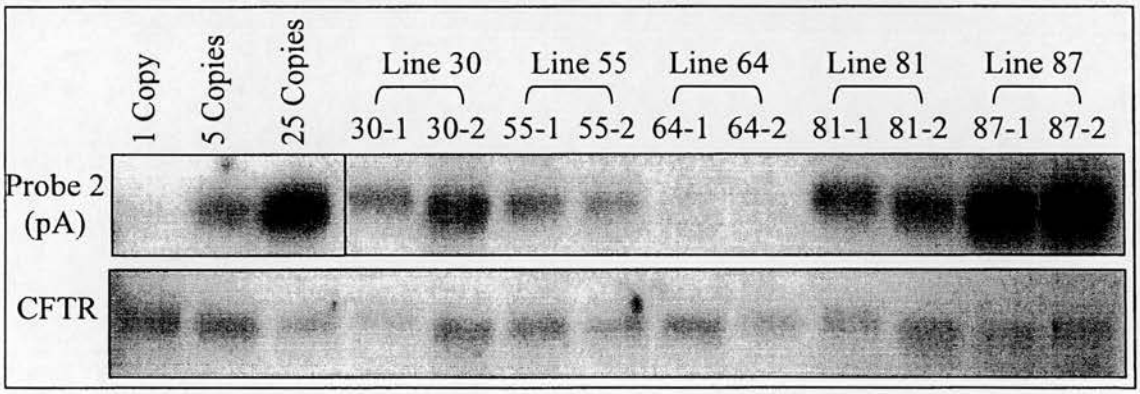



**Figure 4.4 Transgene copy number in the lines of mice.**
Southern blot with 10 μg of *Kpn* I digested genomic liver DNA from 5 independent transgenic lines of mice carrying the 6.95 kb transgene (two mice from each line). The blot was hybridised with probe 2 (Figure 4.1), then stripped and rehybridised with a murine cystic fibrosis transmembrane-conductanse regulator (CFTR) probe as a loading control (lower panel).

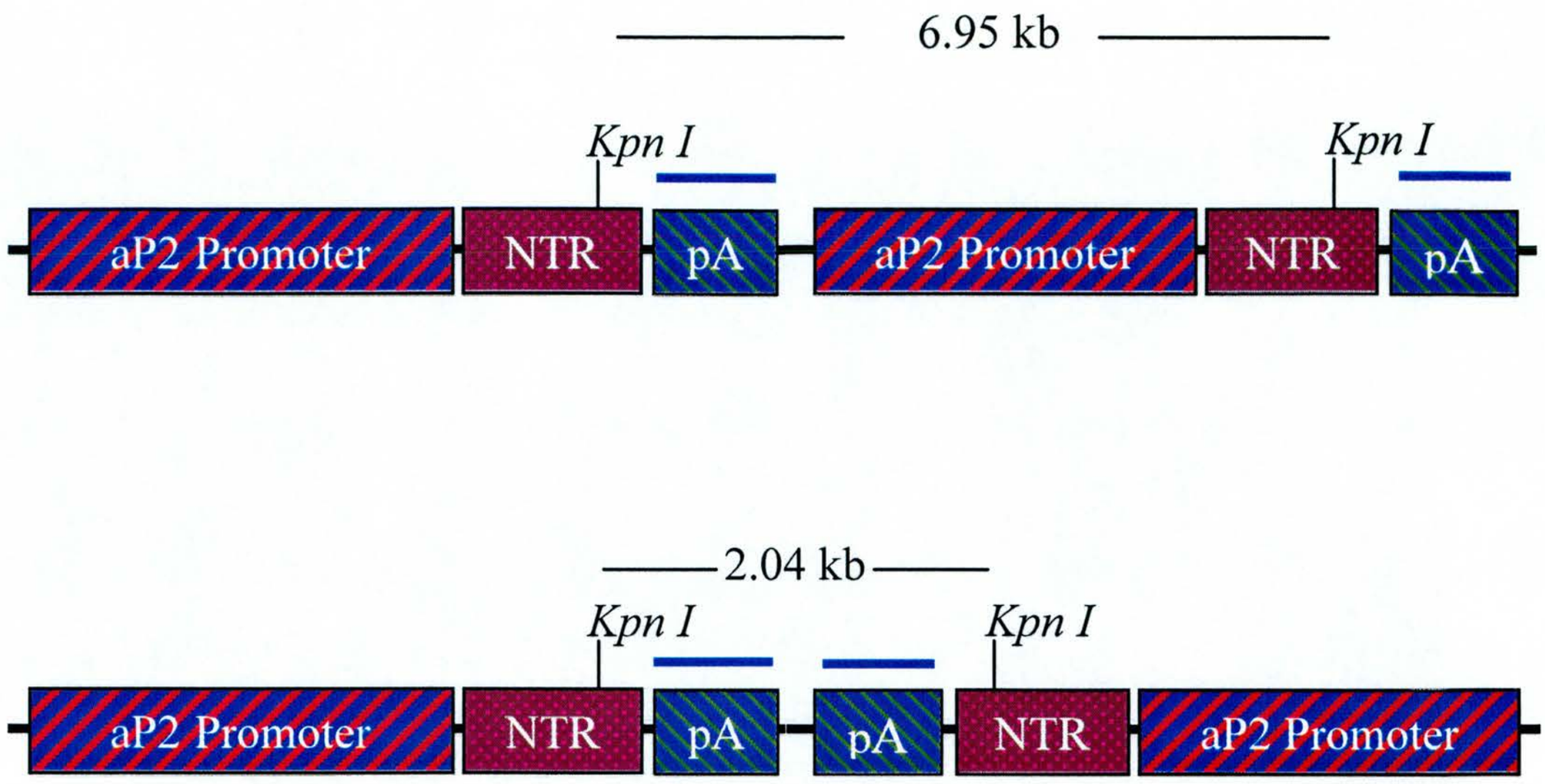



**Figure 4.5 Possible transgene arrays.**
Diagram showing the 6.95 kb band detected when transgene is inserted in a head to tail array and other possible rearrangements.

## 4.6 ANALYSIS OF TRANSGENE EXPRESSION IN THE LINES OF MICE.

### 4.6.1 NORTHERN BLOT ANALYSIS.

Northern Blot analysis was routinely used to determine the expression of the transgene in the different lines of mice. Total mRNA was prepared from epididymal fat pads of male mice and inguinal fat pads of female as in these sites the fat is plentiful and easily accessible. Figure 4.6 shows the result of a Northern blot carried out to some of the transgenic lines that were generated, confirming expression of the transgene in this tissue. Note that an overexposure of the film allows seeing more clearly the low signal from line 64 and 55. NTR mRNA expression was not detected in other transgenic lines generated e.g. lines 34, 39, 42, 47, 67, and 74 (data not shown), which presumably was due to position effects of the transgene.

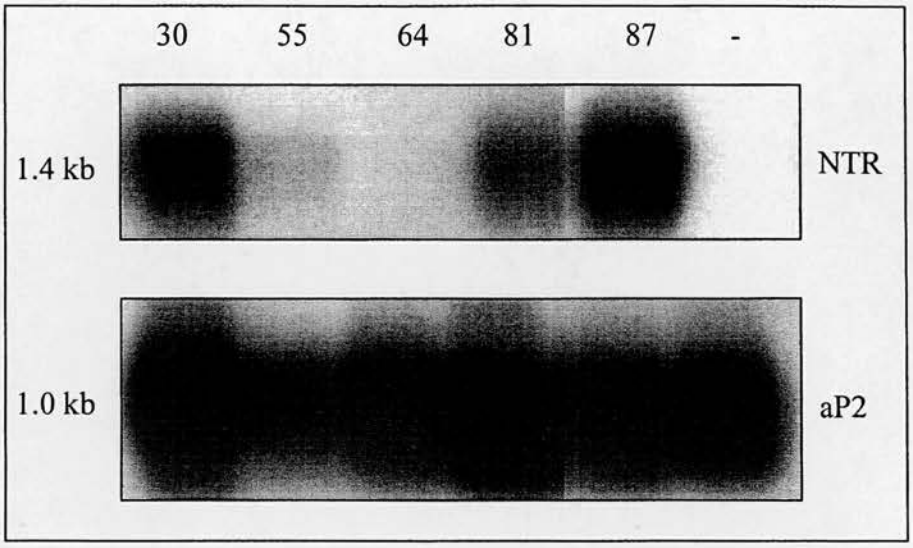



**Figure 4.6 Transgene expression in the different lines of mice.**
Northern blot with 10 μg of RNA from 5 individual samples of each transgenic line. The blot was hybridised with probe 1 (Figure 4.1) in the upper panel and then stripped and re-hybridised with an adipocyte specific probe (aP2) as loading control (lower panel).

### 4.6.2 WESTERN BLOT ANALYSIS.

Levels of protein were also examined in the lines of mice that had shown expression at the mRNA level. Protein extracts were prepared from epididymal fat pads of male and inguinal fat pads of female (as described in chapter 2), run on a 12.5% SDS-PAGE gel, and transferred to nitrocellulose. The Western blot was probed with a polyclonal antibody to NTR. Figure 4.7 shows a single band of approximately 24 kDa (the expected size of NTR) in all 5 lines analysed, which confirm an appropriate translation of the NTR transcript. The same Figure confirmed highest expression of the NTR protein in line 87 followed by lines 30 and 81 and with lower expression lines 55 and 64. A correlation is also observed when the RNA levels of the different lines are compared with their respective protein levels.

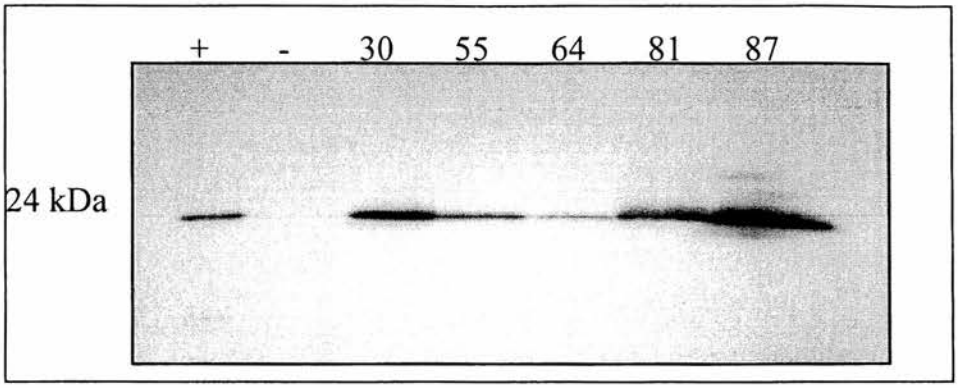



**Figure 4.7 Western blot analysis.**
Total protein tissue extracts (20μg) were prepared from adipose tissue of one member of each of the transgenic lines that showed expression by Northern blot (above). The blot was probed with a polyclonal antibody directed to the *E.coli* NTR.
+: corresponds to a positive control sample (NTR extract of mammary gland from a mouse expressing NTR in this tissue (Clark *et al.* 1997).
-: corresponds to a non-transgenic sample loaded as a negative control.

Table 4.1 Transgenic Lines Generated.

| Transgenic Line | F1 offspring | Transgene Copy Number | Expression levels |
|---|---|---|---|
| **30** | 8/17 (47%) | 5-10 | Medium/High |
| 34 | 8/17 (47%) | N/A | N/D |
| 39 | 8/19 (42%) | N/A | N/D |
| 40 | 0/25 (0)% | N/A | N/A |
| 42 | 4/24 (17%) | N/A | N/D |
| 47 | 3/22 (47%) | N/A | N/D |
| **55** | 4/27 (15%) | ~5 | Medium |
| **64** | 13/20 (65%) | 1 | Low |
| 67 | 10/19 (53%) | N/A | N/D |
| 74 | 10/31 (32%) | N/A | N/D |
| **81** | 3/19 (16%) | 10-15 | Medium/High |
| **87** | 3/38 (8%) | >25 | High |

Number in bold represent the lines that were kept for future experiments.
N/A: Non-analysed; N/D: Non-detected.

### 4.6.3 ANALYSIS OF ECTOPIC EXPRESSION.

The promoter sequences used in this thesis have been demonstrated previously to confer specific expression to the adipose tissue (Ross *et al.* 1990). However, due to the random integration of transgenes is not uncommon to observe aberrant expression in tissues where endogenous genes are not normally expressed. This possibility was assessed by analysing RNA from several tissues including heart, kidney, liver, testis and spleen of one of the transgenic lines that showed high expression levels (line 87) and hybridising this RNA with a probe that detects 1.4 kb band of the NTR transcript (probe 1 in Figure 4.1). As expected, the adipose tissue (as represented by the epididymal fat pad) expresses high levels of the NTR mRNA, however ectopic expression was also observed in some of the other tissues analysed as confirmed by the weak signal that could also be observed in these tissues (Figure 4.8 upper panel). Nevertheless, more detailed analysis of these tissues demonstrated that the hybridisation signal from the kidney RNA is due to the presence of contaminating adipose tissue, since a probe to the endogenous aP2 mRNA also hybridised to this tissue (middle panel in Figure 4.8). However, no such signal could be observed for the spleen, thus suggesting that the signal observed with NTR in this tissue is due to some leaky expression. The nature of this leaky expression is not clear but it might involve effects of the copy number of the transgene, which for this line is >25 (see Figure 4.4 above) or it might be due to effects of the integration site of the transgene.

Another possibility is that the promoter sequences used lack a negative regulatory element that suppresses transcription in the inappropriate tissue. In any case, the former possibilities are more likely since the same promoter sequences have been previously used in a number of transgenic models without ectopic expression in other tissues being reported (Ross *et al.* 1993; Lowell *et al.* 1993; Shimomura *et al.* 1998; Moitra *et al.* 1998).

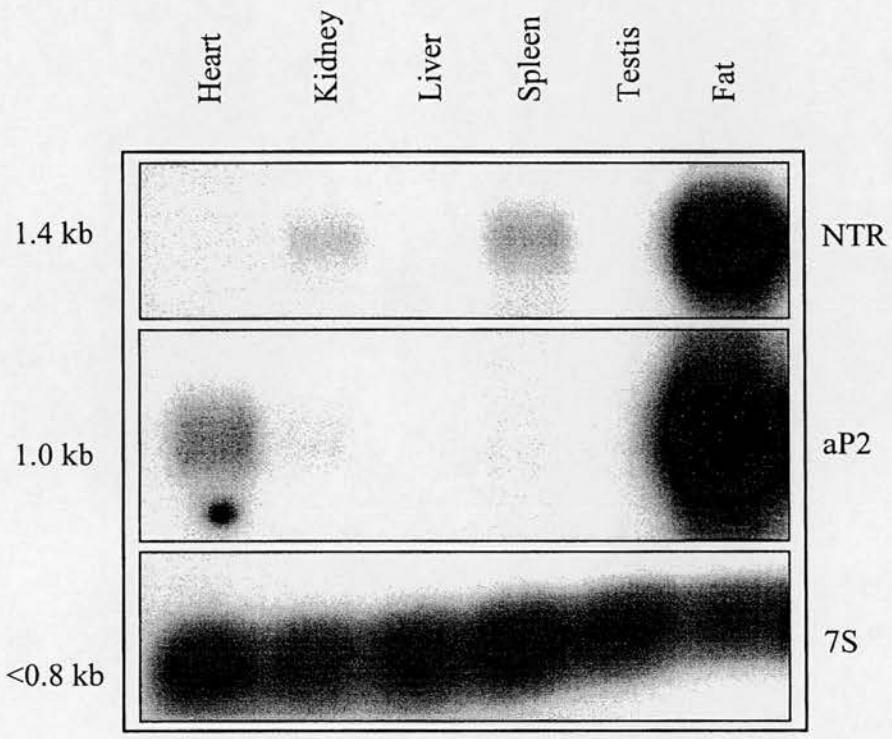



**Figure 4.8 Ectopic expression of NTR mRNA in a high expressing line of mice.** The blot was hybridised with a NTR probe (probe 1 in Figure 4.1) in the upper panel and then stripped and re-hybridised with an adipocyte specific probe (aP2) as control for fat contamination of tissues (lower panel), stripped again and re-hybridised with a 7 S probe as loading control.

### 4.6.4 TRANSGENE EXPRESSION IN DIFFERENT FAT DEPOTS.

Interestingly, from the previous Figure it is possible to observe a strong signal in the heart when the membrane was hybridised with the adipose specific marker aP2 which, indicates that some remaining fat was adhered to this organ (middle panel in Figure 4.8). However, no signal was observed in this tissue when a probe to detect the NTR transgene was used (upper panel in the same Figure). This observation might suggest that different adipose depots express different levels of the transgene. To assess more directly this possibility, a number of adipose depots were chosen to measure the expression levels of the transgene, some of these depots included retroperitoneal, epididymal, subcutaneous and interscapular brown fat. Since endogenous aP2 is expressed in both WAT and BAT it was expected that brown adipose tissue would also express the transgene. Figure 4.9 shows the results of this study confirming expression of the transgene in the brown adipose tissue. The same Figure shows different intensities of the NTR signal in the different adipose depots. In order to assess more accurately the levels of expression from each of these tissues, the radioactivity in the gels was quantified using a phosphorimager, normalised to the aP2 signal and expressed as the fold intensity relative to the mRNA level of the lowest expressing tissue. In this way, it was found that different fat depots have indeed different levels of expression of the transgene, as suggested by the intensity of the NTR signal, with the epididymal fat pads showing the highest levels (1.8 fold) compared to the retroperitoneal that had the lowest. Although one might expect that these subtle differences may fall within experimental error it has long been suggested that different adipose depots may have indeed different functions according to the anatomical location where they are situated. The capacity of inguinal cells to accumulate more triglycerides than adipose cells in the parametrial or retroperitoneal depots is a confirmation of this statement.

Another possibility for this difference might be in the metabolic state of each adipose depot and/or due to effects of the integration of the transgene. Experiments described in the next section confirm this last suggestion.

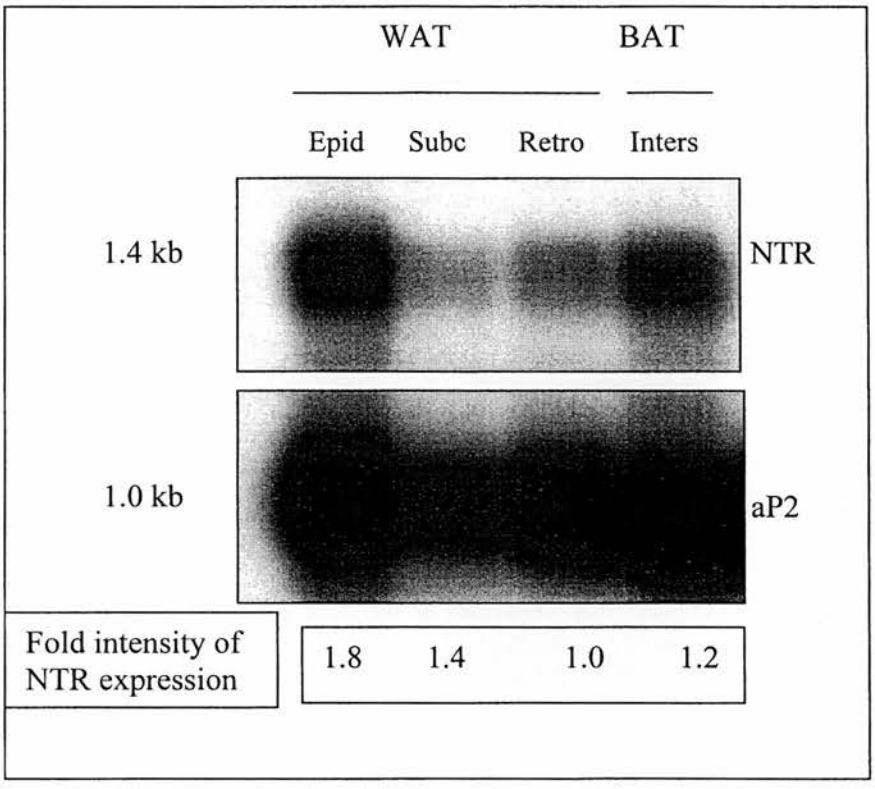
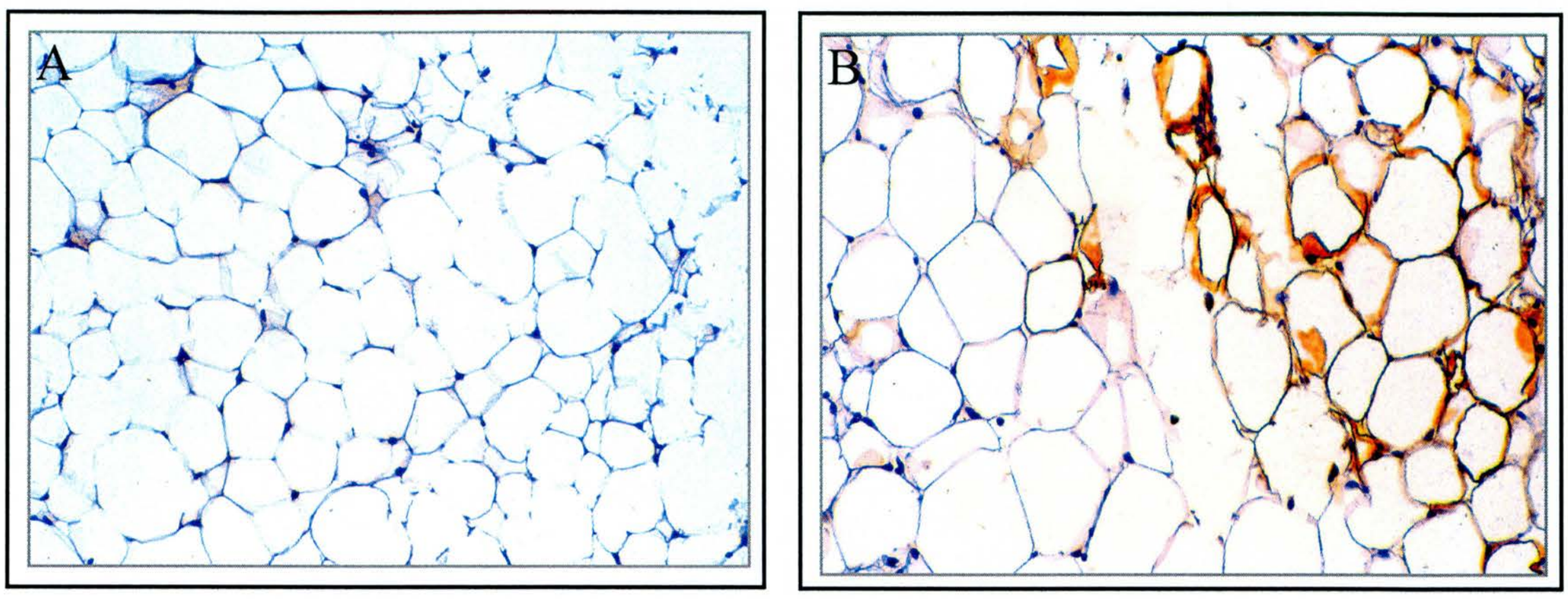



**Figure 4.9 Expression of NTR mRNA in different fat depots.**
The blot was hybridised with a NTR probe (probe 1 in Figure 4.1) in the upper panel and then stripped and re-hybridised with an adipocyte specific probe (aP2) as loading control (lower panel). Epididymal, retroperitoneal, subcutaneous and interscapular brown fat (BAT) are represented.
Intensity of the signal was normalised to the aP2 and expressed as the fold intensity relative to the mRNA level of the lowest expressing tissue.

### 4.6.5 IMMUNOCYTOCHEMISTRY ANALYSIS OF THE TRANSGENE EXPRESSION IN ADIPOSE TISSUE OF TRANSGENIC MICE.

As suggested from the previous experiment different adipose depots were found to express the transgene at different levels, one possibility for this effect was suggested to be due to variegated transgene expression where not all the cells in a tissue express the transgene. Unstable transgene expression has been described previously among progeny of the same founder mouse (Palmiter *et al.* 1984) and it has been suggested that this effect is a property of the transgene integration locus (Dobie *et al.* 1996). Immunohistochemistry analysis was carried out in paraffin embedded sections of epididymal fat pads of two transgenic lines (line 30 and line 87) to assess whether levels of NTR expression were reflected in differential expression of the transgene. Figure 4.10 illustrates the result of this assay in two of the transgenic lines analysed. Adipose tissue sections from these two lines showed clusters of cells strongly expressing the NTR protein, surrounded by non-expressing (silenced) cells (Figure 4.10 B and C) confirming a patchy expression of the NTR.

Variegated transgene expression has been previously observed between individuals of the same transgenic line and it has been suggested that this effect is a consequence of the integration site of the transgene and the number of transgene copies (Dobie *et al.* 1996). In fact, both lines studied in these experiments that showed mosaic expression had a high copy number of the transgene (>10 and >25 for lines 30 and 87, respectively). This mosaic expression may have important implications as suggested by Dobie *et al*, (1996) in biotechnological applications, such as the production of proteins of biomedical interest in the milk of transgenic livestock. Similarly, this mosaic expression may have an important role in gene addition experiments, such as the experiments proposed in this thesis, because variegated transgene expression in these experiments may prevent 100% ablation of adipose cells and therefore result in variable numbers of cells remaining in the tissue.



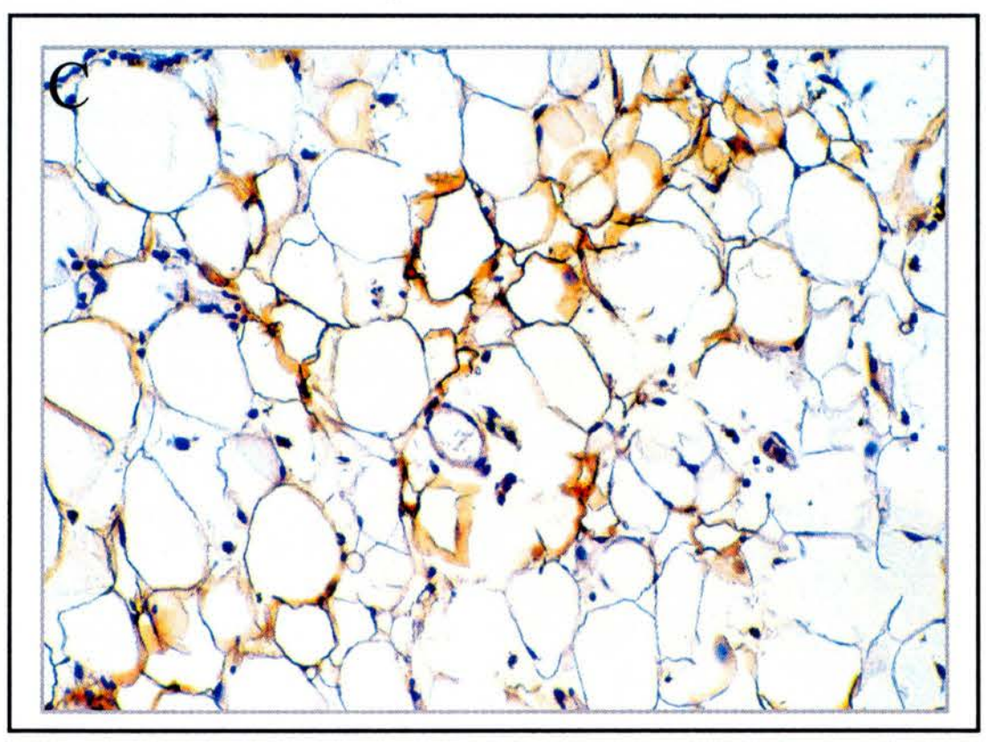




**Figure 4.10 Variegated expression of NTR in the adipose tissue.**
Immunocitochemistry of sections of adipose tissue from control (A) and two different transgenic lines (line 87 B and line 30 C).
Paraffin embedded sections were hybridised with anti serum to NTR. Note the patchy staining of the transgenic sections. Magnification: 200X A-C.

## 4.7 CONCLUSION.

Microinjection of the aP2-NTR transgene (by Roberta Wallace) into single cell mouse embryos resulted in the generation of 12 transgenic founder mice from a total of 96 analysed, an efficiency of 13%, which compares favourably with the efficiency observed by other groups (Boyd and Samid, 1993). All founders showed variable copy numbers of the transgene. 11 founders transmitted the transgene into the offspring albeit the variable rate and one did not generate transgenic offspring presumably due to mosaic effects (Whitelaw *et al.* 1993). All 11 lines of mice had approximately normal litter sizes (i.e. 8-12 pups per litter), and no obvious phenotype was observed as result of the transgene integration.

Expression of the NTR transgene could be observed in 5 out of 11 F1 lines generated, thus indicating that aP2 promoter used contain all the necessary regulatory elements to drive expression of exogenous genes into adipocytes *in vivo*. High levels of expression were observed in some of the lines generated (lines 87, 81 and 30) and these levels were correlated to the protein level. Expression levels of the transgene were far from the levels observed for the endogenous aP2, which might suggest that the promoter sequences used still lack of one or more regulatory sequences necessary for a stronger level of expression in this tissue. Another possibility is some silencing effects from neighbouring chromatin, which might also explain the complete absence of expression in other transgenic lines generated.

Appropriate translation of this prokaryotic protein was also demonstrated thus indicating that eukaryotic cells (adipocytes) have no major problems in processing this E.*coli* NTR sequence. However, a mosaic pattern of transgene expression was observed in the transgenic lines analysed, which may have some important consequences in the proposed ablation experiments with CB1954 as described in the next chapter.

# 5. A MOUSE MODEL TO TEST THE EFFICACY OF CB1954 MEDIATED ABLATION *IN VIVO.*

## 5.1 BACKGROUND.

White adipose tissue (WAT) is the principal site in the body for energy storage, using triglycerides as a remarkably efficient storage mechanism. WAT also helps to regulate energy homeostasis (including food intake, metabolic efficiency, and energy expenditure) via its secreted hormone, leptin and additional hormones. The large variation in fat mass is unlike that of other tissues in the body and is determined both by an individual's genetic background and by environmental factors including diet and physical activity (Comuzzie and Allison, 1998). Excess of body fat, or obesity, is a major health problem, particularly in Western societies, increasing the risk of Type 2 diabetes, hypertension, hyperlipidemia, and coronary artery diseases (Pi-Sunyer FX, 1993). Genetically fat mice exist and have contributed greatly to the understanding of obesity. However, the mechanisms by which obesity causes the associated disorders are unknown. Thus, whole animal models are required for the understanding of these questions because obesity and diabetes are the integrated results of signalling and metabolism in brain, liver, muscle and pancreas, in addition to adipose tissue. Although a number of mouse models with either increased or decreased amounts of adipose tissue have been reported, no mouse model from either knockout or transgenic technologies had been described, at the time of the start of this thesis, that were devoid of fat throughout development, which may imply that fat was essential for life. Thus, the availability of a mouse model where adipose cells could be removed at different stages of mouse development would undoubtedly serve to better understand the physiology of this tissue and more importantly for the study of the associated disorders of obesity.

Chapter 3 showed that differentiated 3T3L1 cells stably expressing the NTR gene can be successfully killed in the presence of the prodrug CB1954. Having assessed the susceptibility of these cells to the prodrug, the next step in this project was to demonstrate that the NTR/CB1954 system works in an animal model *in vivo.*

The previous chapter described the generation and characterization of a number of transgenic lines expressing NTR in adipose tissue, thus confirming the appropriate expression and translation of this prokaryotic protein. This chapter now describes the use of this novel approach of ablation to genetically remove specific cells in an animal. This system was used to ablate adipose tissue cells in an inducible manner through different stages of the mouse development. Upon prodrug treatment, depletion of adipose cells in transgenic mice was observed whereas other tissues were normal and comparable with non-transgenic treated or untreated control mice. The effects and the consequences of the treatment with the prodrug are described.

## 5.2 NTR MEDIATED ABLATION OF ADIPOCYTES *IN VIVO*.

### 5.2.1 PRELIMINARY EXPERIMENTS.

Preliminary ablation experiments were carried out with one of the transgenic lines (line 64), which had showed high levels of NTR in the adipose tissue. Unfortunately, it was later found that this line contained double integration site of the transgene (data not shown). This meant that the offspring had two different patterns of transgene expression, one group with low expression and another group with high expression, depending on the integration site of the transgene. By the time this was discovered, one of the males with low expression levels had already been used to propagate the line and thus all the subsequent generations of this mating had low expression. This was discovered just after ablation experiments had been carried out with this line, thus explaining what little effect that CB1954 had, in terms of ablation of the adipose tissue, in this transgenic line. Northern and Western blot analysis carried out with this line (Figures 4.6 and 4.7 in previous chapter), suggested that this line turned out to be the lowest expressing line from all 5 lines chosen to propagate. Therefore, subsequent ablation experiments most of which will be described in this chapter, were carried out with line 87, which showed high expression levels of NTR by both assays.

Ablation experiments were normally carried out with mice housed at 20°C in individual cages with free access to food and lights on for 12 h/day. Transgenic and

non-transgenic littermates were injected intra-peritoneal (i.p.) with the prodrug CB1954 suspended in arachis oil containing 10% acetone. Toxicity studies on CB1954 carried out by Workman *et al.* (1986) established acceptable doses for single and multiple doses administration in BALB/c and C3H/He mouse strains. Therefore, preliminary studies were undertaken using multiple dose regimens of 40 mg/kg of body weight that were somewhere between the $LD_{10}$ of BALB/c and C3H/He mice. This multiple dose regimen included, most typically, daily injections for 3 days although 5 injections were also used in other experiments.

To characterise the mice, a number of tissues were dissected to assess whether ablation had occurred. These tissues included the gonadal fat pad since it is plentiful and can be dissected easily from surrounding tissues. The skin was also assayed since this contains a well-defined structure and white adipose tissue begins to appear soon after birth; liver was analysed to control for any secondary toxic effect of the prodrug. Organ weights were also taken to monitor the effects of the ablation of adipocytes or indirect effects of the prodrug in other tissues.

### 5.2.2 ABLATION EXPERIMENTS WITH LINE 87.

Ablation experiments with this line involved transgenic and non-transgenic littermate mice of 7 weeks old that were injected intraperitoneally with CB1954 for 3 days. Another group of mice was left untreated to serve as control. An example of a dose regimen of 40 mg/kg of body weight used is shown below in Table 5.1. The weight of the mice was routinely monitored daily starting on injection day until the mice were sacrificed. The first experiment was carried out with males that received a dose regimen of 40 mg/kg of body weight. At this dose, transgenic mice, but not non-transgenic control mice, showed loss of condition. These mice were observed to be cold, hunched and reluctant to move immediately after the second injection. Unfortunately, no device was available, at that time, to measure body temperature. Therefore these mice were humanly sacrificed on the second day in conformity with the Animals (Scientific Procedures) Act 1986, and the carcasses sent to Roslin Nutrition for analysis of the total body lipid content, which was carried out as described in chapter 2. Unfortunately, this analysis showed no major difference in

the content of fat, presumably due to the short time that the mice were kept alive, thus not giving enough time to the prodrug to act. These experiments suggested the need of heated pads to keep the mice warm, for which the necessary amendments to the Project License were made to allow the execution of these experiments.

Table 5.1 Dose-regimen of injections

| **Injections: FEMALE CB1954 (4mg/ml)** | | |
|---|---|---|
| **Body weight** | **injection volume** | **CB1954** (each/total) |
| 20-25g | 0.2ml | 0.8mg/2.4mg |
| 26-30g | 0.25ml | 1.0mg/3.0mg |
| >30g | 0.3ml | 1.2mg/3.6mg |

After approval of the amendments to the Project License, a different experiment was carried out this time with females that received a similar dose-regimen of 40 mg/kg of body weight. A drop in the body weight of mice that have received treatment with CB1954 was observed (transgenic and non-transgenic mice injected with the prodrug). More than half of the experimental mice started to lose weight on day 2 of the experiments, and after withdrawal of the injections they showed a reduction of 10-15%, suggesting that the threshold of toxicity was reached (Figure 5.1 A), although this effect was more pronounced in transgenic mice with some of them losing as much as 23% of body weight towards the end of the regimen.

A reduction in the body weight was also observed in a different experiment with males and females that received a lower dose of 20 mg/kg of body weight. With this regimen, transgenic and non-transgenic mice treated with the prodrug lost approximately 4 and 8% of body weight, respectively (Figures 5.2 A and B), a much smaller reduction compared to the regimen of 40 mg/kg of body weight used with females.

In general, weight loss due to enteritis would be the first sign of toxicity, as previously described in rats (Cobb LM 1970) and mice (Workman *et al.* 1986). A loss of body weight has also been observed in other experiments involving mice that have been injected with CB1954 (Drabeck *et al.* 1997; Cui personal communication) and it has been suggested that this effect is partly due to the effects on NTR positive bacteria in the gut (Clark *et al.* 1997).

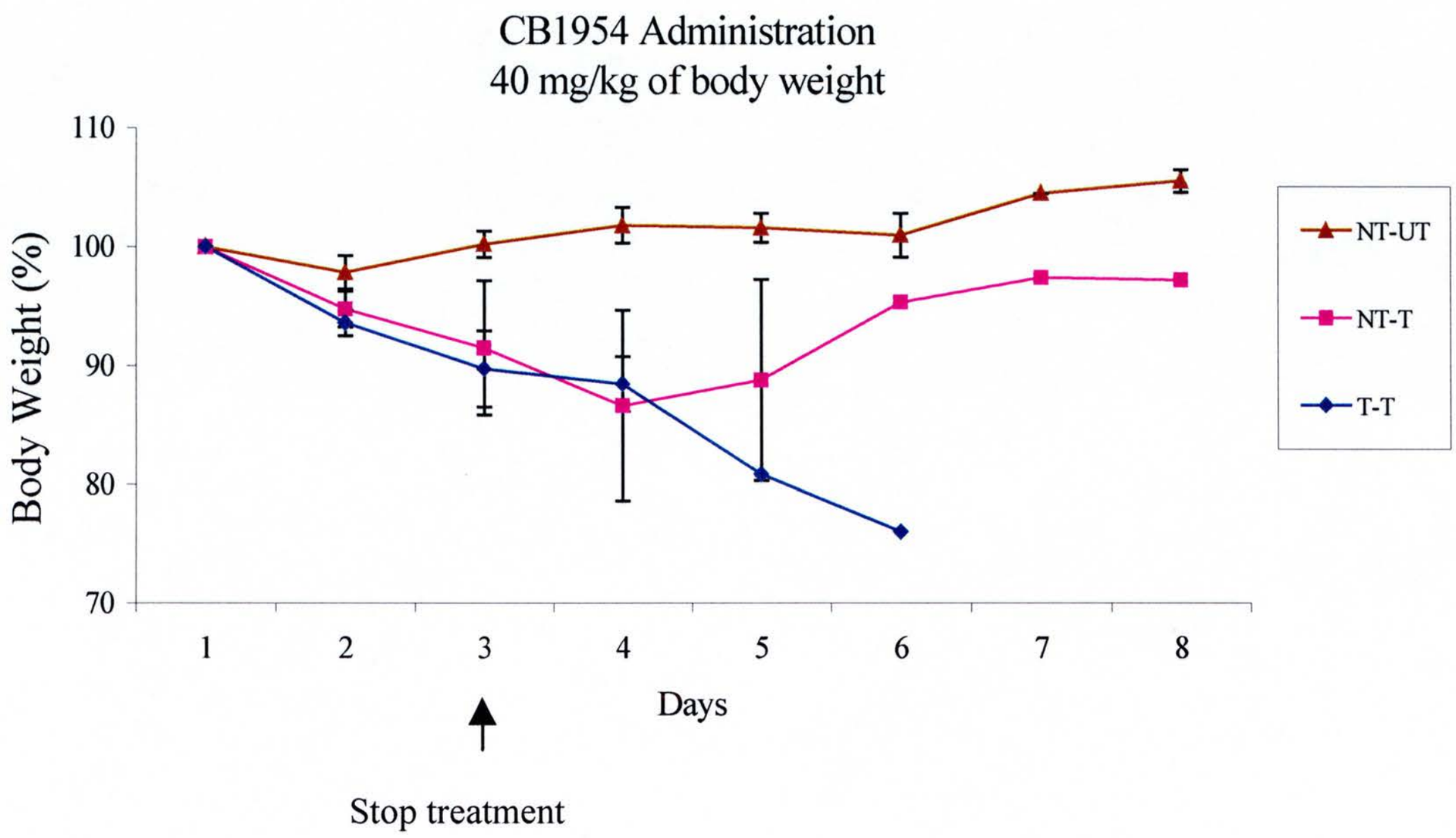
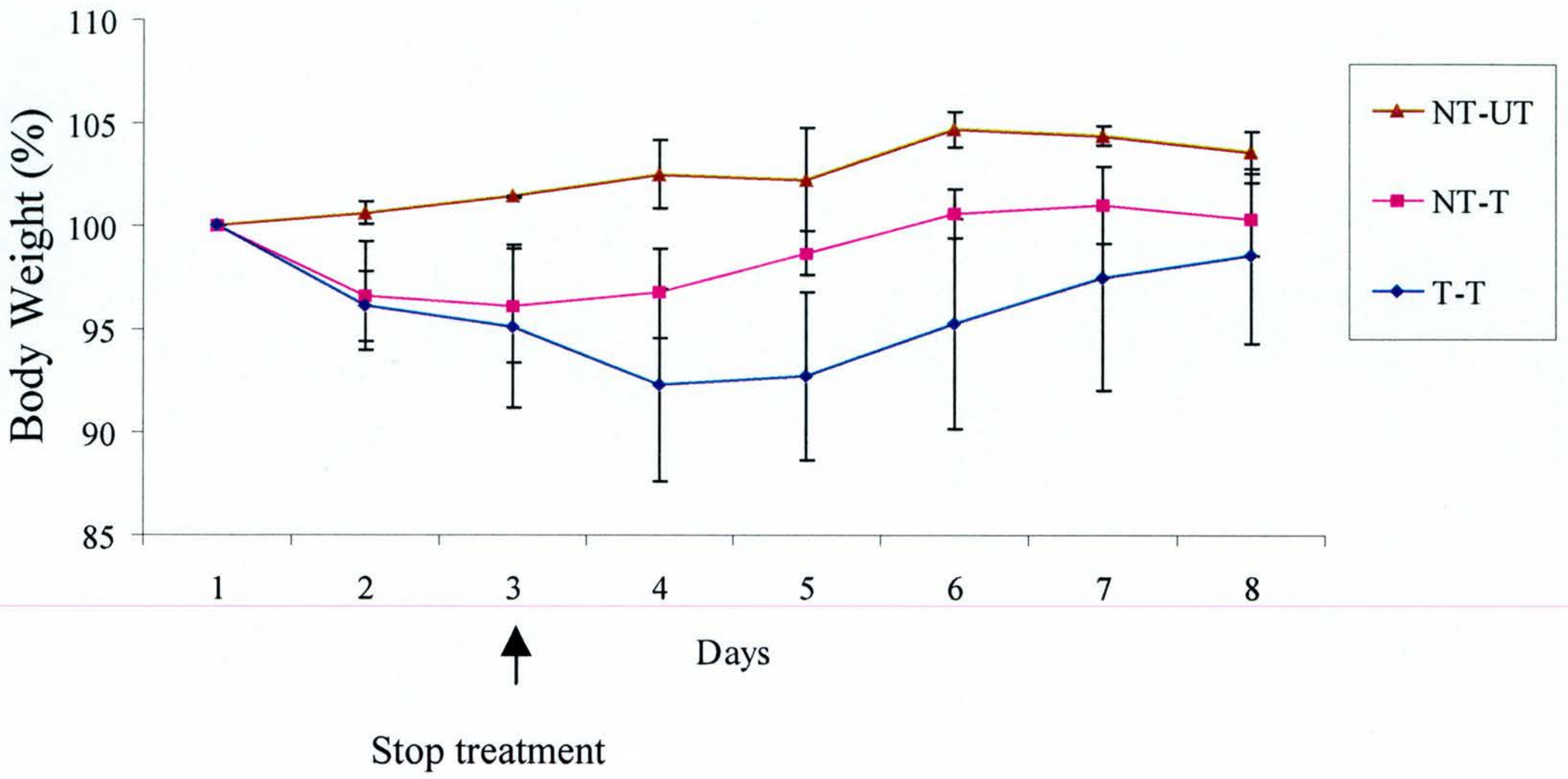



**Figure 5.1 Measurement of body weight during treatment with CB1954.**
7 week old transgenic (T) and non-transgenic (NT) littermate mice were injected (T) with CB1954 at doses of 40 mg/ kg of body weight for 3 days. Another non-transgenic group was left untreated (NT-UT).

A




B

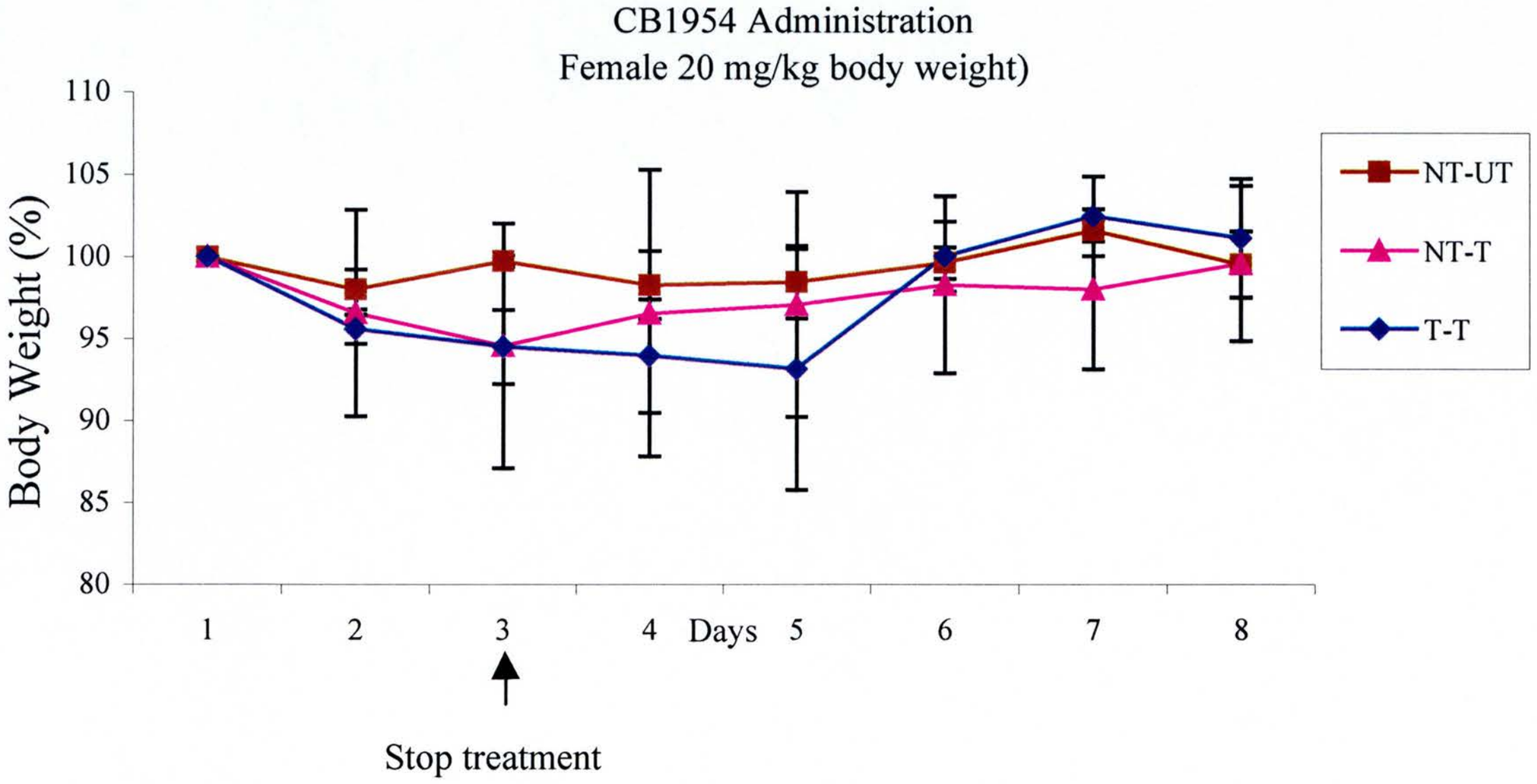



**Figure 5.2 Measurement of body weight during treatment with CB1954.**
7 weeks old transgenic (T) and non-transgenic (NT) littermate mice were injected (T) with CB1954 at doses of 20 mg/ kg of body weight for 3 days. Another non-transgenic group was left untreated (NT-UT)
A) Male, B) Female.

There was another effect of the treatment with the prodrug, which was observed only in the transgenic group that received this higher dose of 40 mg/kg of body weight (females). These mice were found to be hunched, cold and reluctant to move after treatment with the prodrug. When body temperature was measured, this time with a rectal thermometer, a considerable drop in approximately 5°C (32°C±0.5) compared to the control groups (37°C±0.6) was observed. A drop in the body temperature has also been observed previously in mice devoid of fat (Moitra *et al.* 1998). This drop in body temperature was suggested to be due to the effects of the loss of fat, which is needed for heat production. This group described that husbandry of fat depleted mice was not trivial and they required heated pads to keep the mice warm. Nevertheless, mortality of approximately 60% was observed at weaning of these mice.

A similar device was used in these transgenic mice treated with CB1954. Unfortunately, heated pads did not improve the conditions of the mice, albeit a slight recovery in the body temperature that was observed, which meant that these mice were humanly sacrificed at the end of the day (day 3 of the injections for this experiment). A similar effect was observed in other two transgenic mice in this group although this effect was observed later than the first group (day 5 and 6). Another transgenic mouse was found dead on day 6 and was not included in the analysis. The cause of death is unclear but it might be a direct consequence of the ablation of adipose cells or a secondary toxic effect of the prodrug. In contrast to transgenic mice, non-transgenic mice injected with the prodrug did not show this effect and the only effect of the prodrug observed in these mice was a drop in the body weight as already discussed above.

Earlier toxicology experiments carried out by Workman *et al.* (1986) also showed a marked reduction in the body weight of up to 30% at non-lethal doses of CB1954. At higher doses, toxic symptoms developed towards the end of the regimen as described by these authors. These symptoms included shaking, splaying of the hindlimbs, and walking on tiptoe, all indicative of neurotoxicity. In the experiments described in this section neither transgenic nor non-transgenic mice treated with CB1954 showed these symptoms.

Transgenic mice that received a lower dose of 20 mg/kg of body weight showed no signs of morbidity with this regimen and there was no need to sacrifice them earlier than planned. Despite the lost in body weight, these mice (transgenic and non-transgenic injected with CB1954) tended to recover their body weight almost immediately after withdrawal of the injections with this effect being more pronounced in the transgenic group that had lost more weight than non-transgenic group injected with the prodrug (Figure 5.2 A). The same effect was also observed in another experiment involving females that had received this lower dose of 20 mg/kg of body weight (Figure 5.2 B). Interestingly, despite having lost more weight than the control group (~6%), transgenic females recovered their weight and eventually weighed more after these 7 days of prodrug free period. It might be suggested that this recovery in the body weight has a major component in leptin levels falling down in parallel to the adipose stores. This would derive in an increased food intake and decreased energy expenditure as leptin acts as a feedback loop to maintain constant stores of fat (Flier, J.S 1997). Although food intake was not measured in these experiments, confirmation of a possible involvement of leptin will come from another experiment were leptin levels where directly measured in the plasma of transgenic mice after the treatment with CB1954, as will be described in the next chapter.

### 5.2.3 ANATOMICAL CHARACTERISTICS OF TRANSGENIC MICE AFTER TREATMENT WITH CB1954.

Autopsy of these mice revealed a considerable reduction in the size of the adipose stores in the transgenic groups, with this effect being more pronounced in the group of females that received the higher dose of 40 mg/kg of body weight. In fact, three of these mice that were sacrificed earlier after been in the heated pads, showed no visible adipose mass in any of the fat depots analysed, including parametrial (gonadal), retroperitoneal and inguinal. The inguinal and parametrial fat pads of two other mice that were sacrificed later (day 4 and day 5 in Fig 5.1) were significantly reduced ($P<0.001$), weighing only 20 and 40% of control mice, respectively (Figure 5.3 A and Table 5.2 A). The interscapular brown adipose tissue

(BAT) in transgenic mice were also reduced in size, although this effect was not significant due to the high variation that was observed between individuals of this group. In contrast, non-transgenic mice injected with CB1954 had comparable levels of adipose mass to the control untreated group albeit a slight reduction that was observed in the inguinal fat mass of this group that might indicate an indirect effect of the prodrug concomitant with the reduction in body weight.

Transgenic males that received a lower dose of 20 mg/kg of body weight also showed a considerable reduction of the adipose stores (Table 5.2 B) although this effect was not as marked as in the female group that received 40 mg/kg of body weight. The epididymal fat pads of transgenic mice weighed on average 60% less (P=0.01) than the control untreated group (Figure 5.3 B and Table 5.2 B). A small reduction was also observed in the non-transgenic treated group but this reduction was not significant when compared by a Student *t* test with the untreated group (data not shown). A small reduction of fat was also observed in the inguinal fat pads of females in the experiment described above. This effect might be due to normal variation that may be observed between mice in whose case it should disappear if a larger population is included in the analysis. Another possibility is an indirect effect of the prodrug concomitant with the reduction in body weight that is observed in these mice after treatment with CB1954. Retroperitoneal fat pads of transgenic mice were also significantly reduced by 50% (P=0.01) compared to the levels of the other two control groups (Table 5.2 B).

Comparison of the other organs showed a reduction in the size of the spleens for both groups (transgenic and non-transgenic) injected with CB1954 at this higher dose of 40 mg/kg of body weight. A possible explanation for this effect has been previously suggested by Drabeck *et al.* (1997). Since the prodrug is administrated intraperitoneally, the spleen is accessible almost immediately after injection and will experience a much higher local concentration of prodrug than other organs. Another possible explanation is some leaky expression of the transgene in the spleen, thus explaining the smaller size in the transgenic group compared with the non-transgenic group treated with the prodrug (Table 5.2 A).

Table 5.2 A Body composition of females injected with 40 mg/kg of body weight.

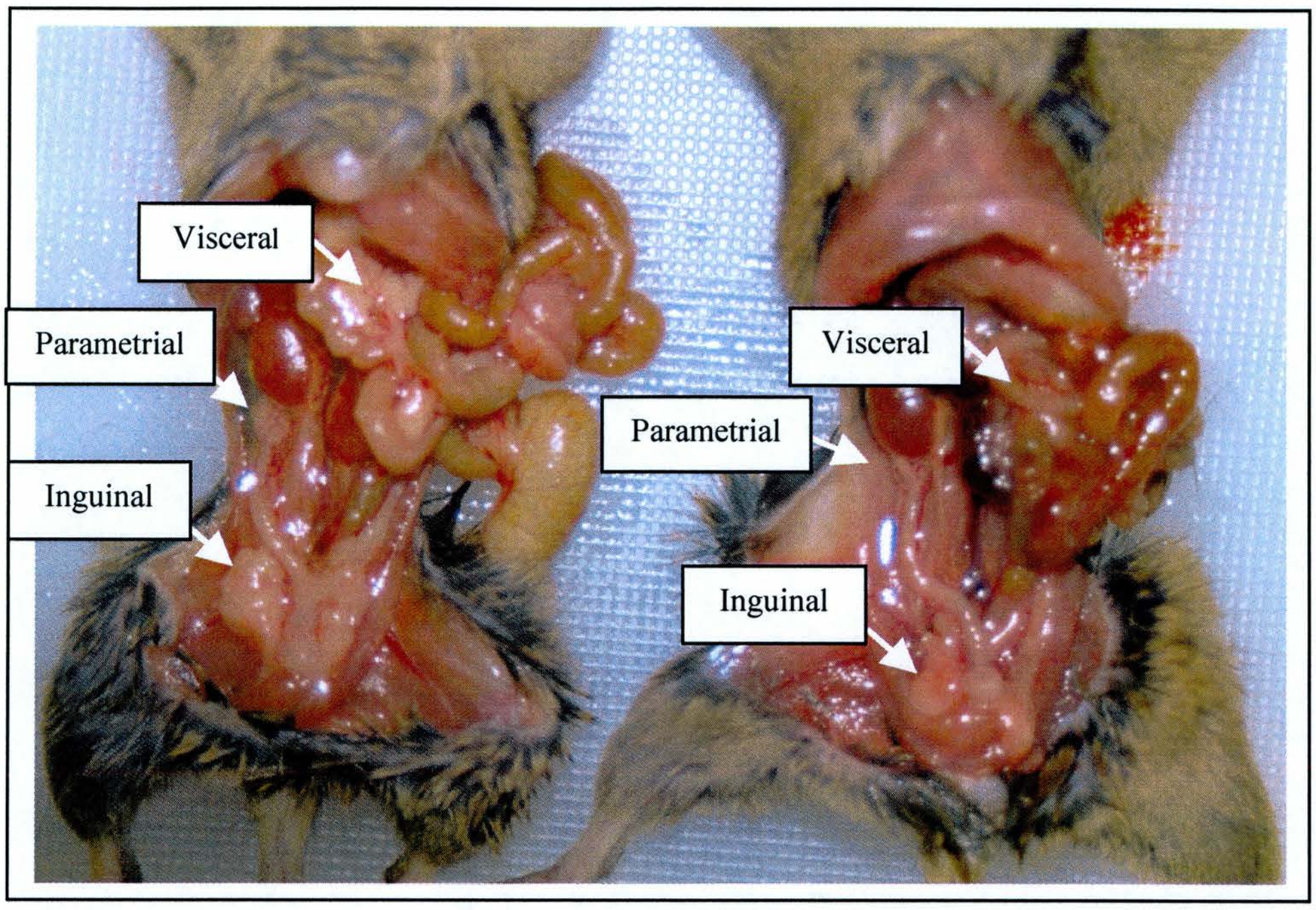
| | Body Weight Grams | Gonadal (WAT) (%) | Inguinal (WAT) (%) | Intersc. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| T-T (n=4) | 15.6±0.6 * | 0.07±0.01 Ψ | 0.08±0.01 Ψ | 0.18±0.05 | 4.50±0.1 | 0.77±0.03 | 0.20±0.01 * | 0.52±0.05 |
| NT-T (n=2) | 15.1±0.3 * | 0.17±0.01 | 0.32±0.05 | 0.27±0.01 | 4.70±0.7 | 0.70±0.05 | 0.25±0.01 * | 0.56±0.02 |
| NT-UT (n=3) | 18.0±0.4 | 0.15±0.01 | 0.42±0.02 | 0.23±0.04 | 4.60±0.2 | 0.71±0.06 | 0.34±0.05 | 0.58±0.02 |
| P Value | *=<0.05 | Ψ=<0.0001 | | | | | | |

Table 5.2 B Body composition of males injected with 20 mg/kg of body weight.

| | Body Weight Grams | Gonadal (WAT) (%) | Retro (WAT) (%) | Interscap. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| T-T (n=3) | 24.5±1.1 | 0.27±0.01 * | 0.07±0.01 * | 0.19±0.03 | 5.45±0.1 Ψ | 0.69±0.03 Ψ | 0.24±0.02 | 0.52±0.02 |
| NT-T (n=3) | 23.8±0.7 | 0.51±0.07 | 0.13±0.01 | 0.15±0.01 | 4.86±0.2 | 0.78±0.01 | 0.27±0.02 | 0.49±0.01 |
| NT-UT (n=2) | 26.0±1.3 | 0.64±0.04 | 0.13±0.01 | 0.15±0.05 | 4.70±0.1 | 0.81±0.04 | 0.29±0.03 | 0.54±0.03 |
| P Value | *=<0.01 | Ψ=0.06 | | | | | | |

Data are mean ± S.E.M. Animals were transgenic and non-transgenic littermate of 7 weeks old. The organ weights were measured as a percentage of the animal's body weight. Probabilities (P) are from ANOVA one way. Groups with significant differences are shown.
T-T: Transgenic mice treated with CB1954, NT-T: Non-transgenic mice treated with CB1954, NT-UT: Non-transgenic mice untreated.

A)




B)

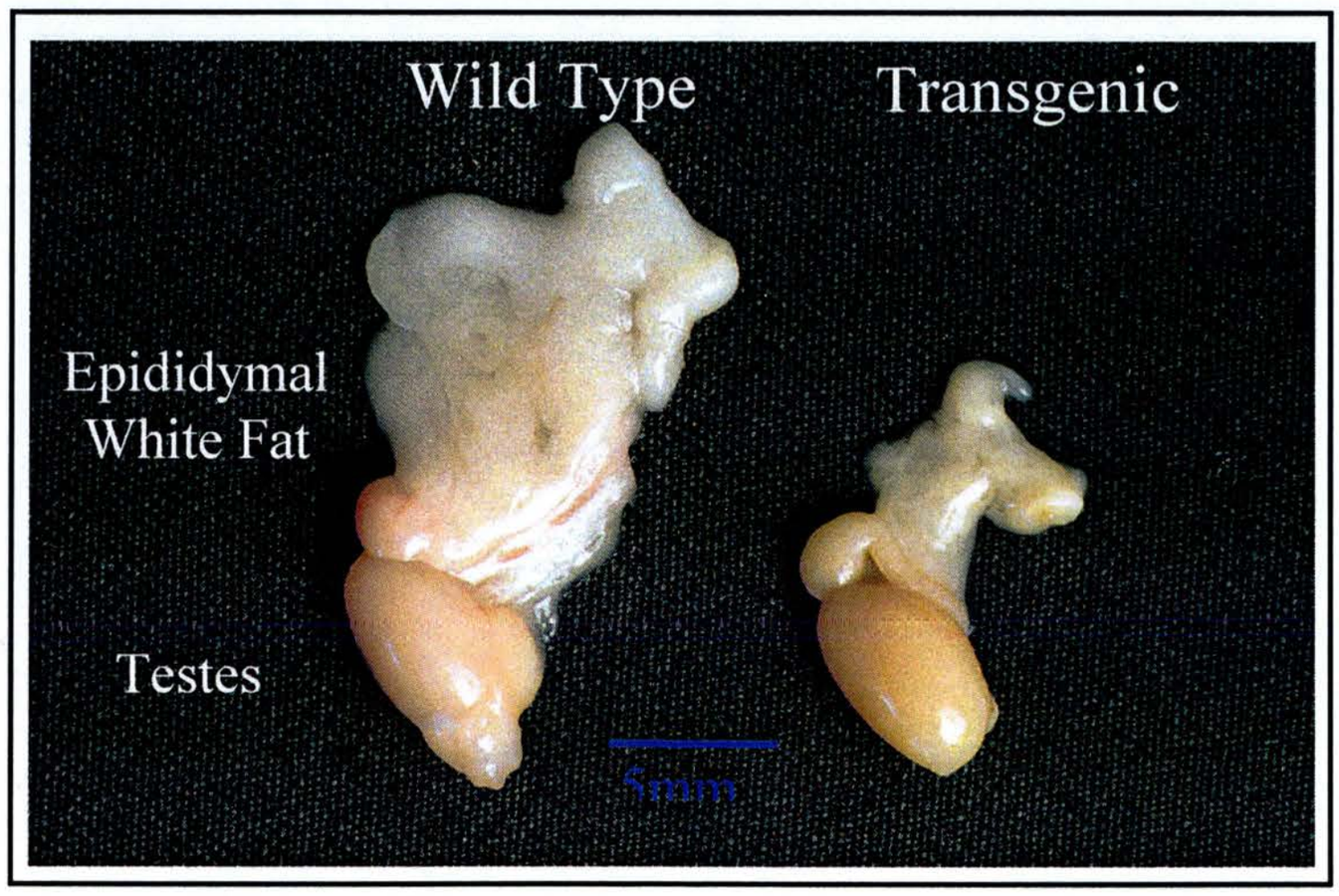



**Figure 5.3 Appearance of fat depots in wild type and transgenic mice treated with CB1954.**

A) Peritoneal cavity showing the different fat depots of a wild type (left) and transgenic littermate (right), age 7 weeks old, after treatment with CB1954.

B) Photomicrograph of testes showing the reduction of fat in the epididymal fat deposits of the transgenic mouse, compared to the wild type control.

Interestingly, toxic effects in the spleen was not observed at this lower dose of 20 mg/kg of body weight (Table 5.2 B), as the size of the organ was comparable to the size of control groups. Visual inspection of other tissues showed no great difference suggesting a specific effect in the adipocytes. Although, small and large intestines were found distended, possibly confirming the enteritis described by Cobb L. M. (1970) (Figure 5.3 A) with this effect being also correlated to the dose of CB1954 used. This would explain the lack in gain of body weight experienced by the mice while treated with the prodrug.

### 5.2.4 HISTOLOGICAL CHANGES IN THE FAT OF TRANSGENIC MICE TREATED WITH CB1954.

The white adipocyte is characterised by a single large lipid droplet that occupies most of the cytoplasmic space pushing the nucleus to the cell periphery. In contrast, the brown adipocyte has a dense cytoplasm, with multilocular lipid droplets, caused by its high content of mitochondria. Histologic sections of the inguinal fat pads in control mice, stained with hematoxylin and eosin, showed mature adipocytes of uniform size each of which contained a characteristic unilocular fat droplet (Figure 5.4 A). In contrast, remaining inguinal fat pads of transgenic female that received a dose of 40 mg/kg of body weight contained a mixture of mature and immature adipocytes with some empty spaces occupied by stroma where adipocytes have disappeared (Figure 5.4 B). Epididymal fat pads of male that received this lower dose of 20 mg/kg of body weight did not show this marked disappearance of adipocytes as was observed with females (not shown) in part due maybe to the lower dose of CB1954 that was used in this group and presumably due to the rapid removal of death cells by the phagocytic machinery, as these mice were sacrificed 7 days after the beginning of the experiments, which is in contrast to the female group that was sacrificed much earlier.

In contrast to wild type WAT, BAT of control mice contained large areas of typical mitochondria rich eosinophilic cells with multiple lipid droplets as well as regions of white fat (Figure 5.4 C). Histological examination of the brown fat in

transgenic females, at this high dose of CB1954 showed some atrophic cells, which were palely stained with the eosin, presumably indicating reduction in the mitochondrial content, and the white fat that covers the brown fat as the mice ages was virtually absent (compare Figures 5.4 C and D). This atrophy of the brown adipose tissue might explain the impaired ability to thermoregulate the body temperature that was observed in these mice, since it is well known the function of BAT in non-shivering thermogenesis, in which fuel oxidation is uncoupled from ATP synthesis to generate heat. One way to demonstrate whether BAT was functional could have been by exposing these mice to cold and measuring the UCP content. It is predicted that if BAT thermogenesis was indeed impaired, the levels of UCP would not increase. Although due to the lowered body temperature that was observed in these mice they may have not been able to withstand the cold. Unfortunately, cold exposure experiments were not permitted in the Project licence.

Analysis of sections of brown adipose tissue in mice that received the lower dose of the prodrug, showed no major histological difference with control groups, which confirms the ability of these mice to cope with this regimen of CB1954 (nor was a reduction of body temperature observed in these mice). Although in sections of some transgenic mice it was possible to observe less eosinophilic staining (compare Figures 5.5 C and D).

Since the aP2 promoter/enhancer is expressed in both WAT and BAT adipose tissues, one might have expected that the BAT would also be ablated. In fact, Northern blot analysis described in section 4.6.4 in previous chapter confirmed expression of the transgene (NTR) in this tissue. Nevertheless, from the histology studies carried out in these mice it seems that only white adipocytes were ablated, although definitive proof of this should come from analysis of the cellular and total protein content of this tissue.

It is possible that a selection exists against BAT ablation, although viable, partially BAT-ablated mice have been produced (Lowell *et al.* 1993). Other two mouse models showed a similar resistance of the brown adipose tissue to the ablation (Shimomura *et al.* 1998; Moitra *et al.* 1998). Both teams genetically blocked the growth of fat cells by altering transcriptions factors that are crucial to cell growth and differentiation. The changes produced by these two teams have

similar effects: The mice were born with little or no white adipose tissue, while the brown adipose tissue was only reduced in size in one case and enlarge with the appearance of inactive brown cells in the other. It is not clear the partial resistance of brown adipocytes to the ablation since the same aP2 promoter/enhancer was used in these studies, but a suggestion given by one of the authors pointed to a homeostatic response of the brown adipocyte to an altered metabolic status of these mice (Moitra *et al.* 1998).

Haematoxylin and eosin staining of sections of the skin showed a considerable reduction of the adipose cells in the sections of transgenic mice and in some occasions there was a complete disappearance of the adipocytes. This effect was especially evident in transgenic females that received the higher dose of CB1954 (Figures 5.4 E and F). Transgenic male with the lower dose also showed a reduction in the adipocytes (Figure 5.5 A and B), although this effect was not as marked as with females at the higher dose. This might be in part due to the lower dose of CB1954 used with males although the control groups showed in general lower number of adipocytes (compare Figures 5.4 E and 5.5 A). This effect might be due to sex differences with female having more fat than male as both group of mice were of the same age. Thus, these results confirmed that ablation of adipocytes was not restricted to a specific fat deposits.

Histological analysis of the livers in transgenic mice showed some diffuse swelling of hepatocytes with disorganization of the hepatic cords and most blood vessels were congested with blood (Figure 5.4 H). This effect was observed with both regimens of CB1954 (20 or 40 mg/kg of body weight) although it was not observed in all mice. This might explain the larger size of the livers that was observed in especially transgenic male with the dose of 20 mg/kg of body weight which, might indicate the effects of the toxic metabolites of CB1954 being metabolised by the liver.

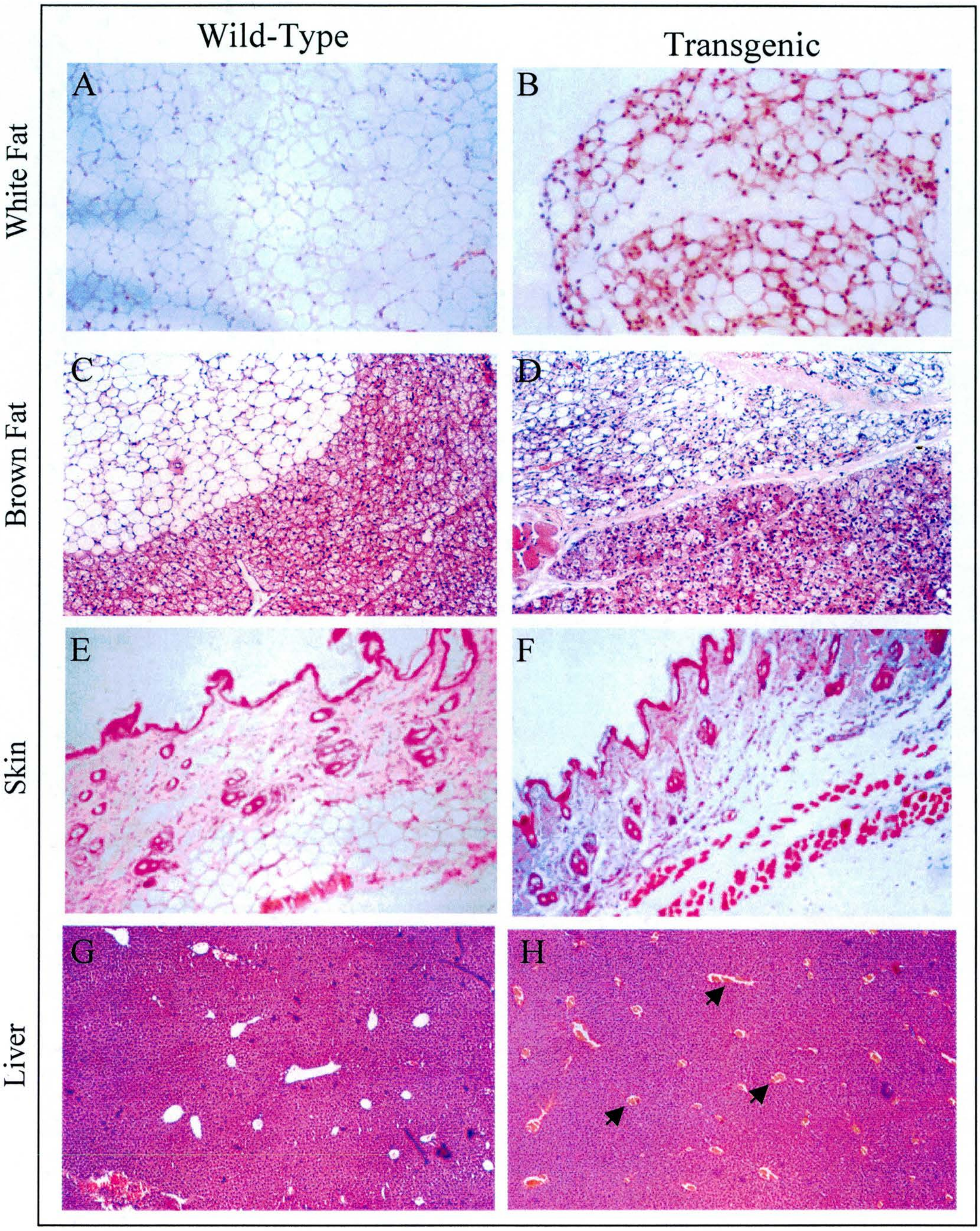



**Figure 5.4 Histology of different tissues after treatment with CB1954.**
Histological sections of white fat (A and B), interscapular brown fat (C and D), skin (E and F), and liver (G and H) from 7 weeks old female transgenic (B, D, F and H) and non-transgenic (A, C, E and G) littermates.
Tissues were fixed in NBF and paraffin-embedded sections stained with hematoxylin and eosin. Magnification: 100X A,B,C, D, E and F; 40X G and H.

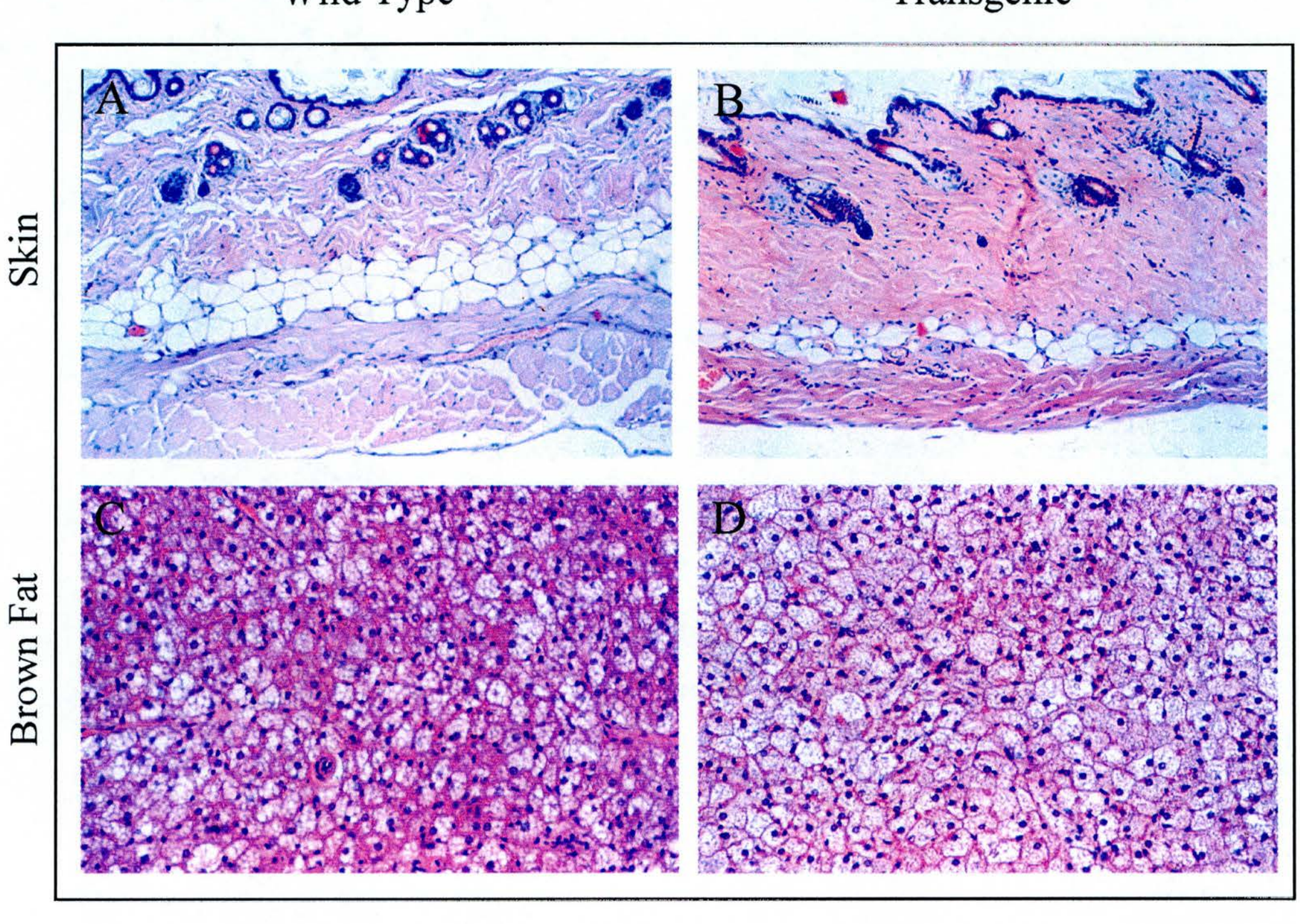



**Figure 5.5 Histology of different tissues after treatment with CB1954.**
Histological sections of skin (A and B) and interscapular brown fat (C and D) of wild type and transgenic mice are shown.
Tissues were fixed in NBF and paraffin-embedded sections stained with haematoxylin and eosin. Magnification: 100X A and B, 200X C and D.

To confirm the *in situ* death of adipocytes after treatment with the prodrug, paraffin embedded sections of remaining inguinal adipose tissue was analysed by the Tunel assay as described in section 3.10 in chapter 3. Figure 5.6 shows the result of this assay demonstrating the presence of some apoptotic cells only in sections of transgenic mice that were treated with the prodrug (panel B), while the control untreated group showed no signal.

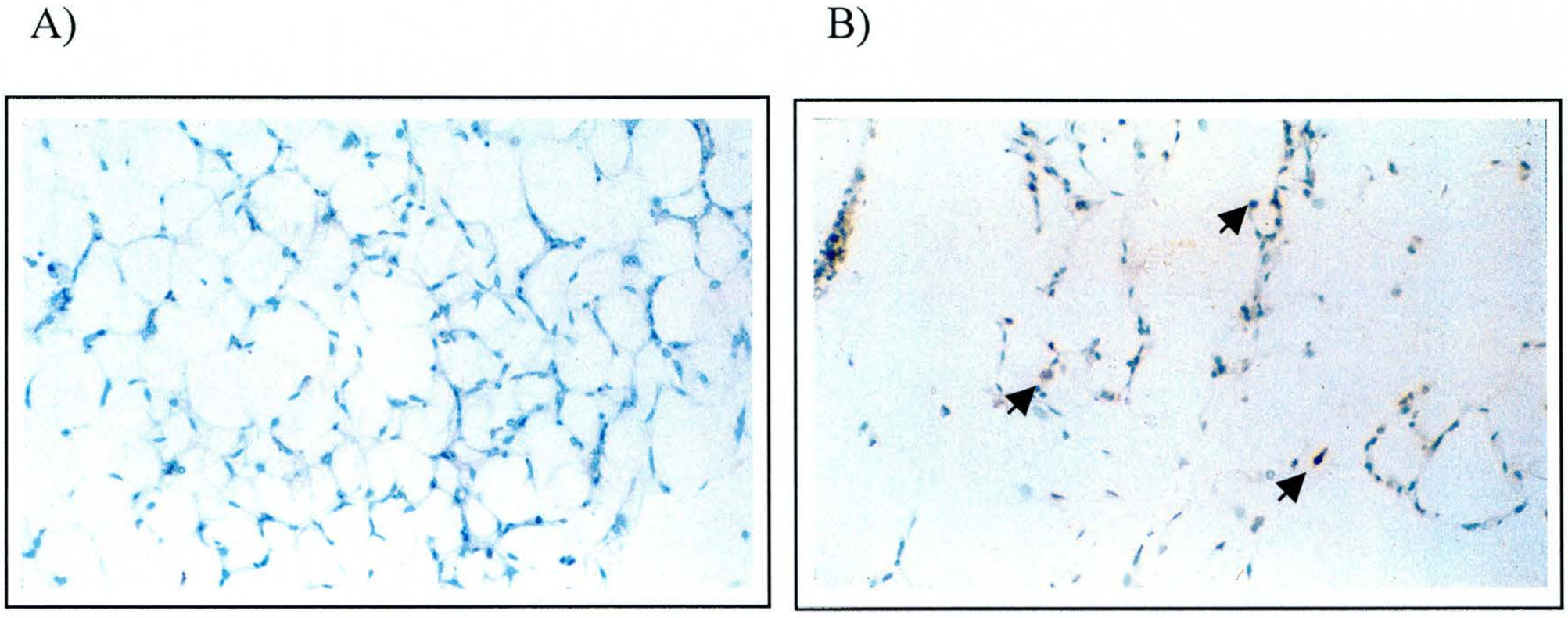



**Figure 5.6 *In situ* Tunel Assay.**
The Figure shows the result of the Tunel assay carried out in paraffin embedded sections of inguinal fat pads of females treated with CB1954 at doses of 40mg/kg of weight. Control non-transgenic treated (A) and transgenic treated (B) are shown. Magnification: 40X

The results shown in the last two sections clearly demonstrate selective killing of adipocytes in transgenic mice expressing NTR in these cells. The magnitude of adipose tissue ablation also correlated to the morbidity effects observed in these mice. While a lower dose of 20 mg/kg of body weight produced a partial reduction of adipocytes without any signs of morbidity. A higher dose of 40 mg/kg of body weight resulted in a complete disappearance of any visible adipose depots in some transgenic mice. This reduction of adipose tissue had important implications especially in the ability to thermoregulate body temperature. Some

morbidity effects could be observed after 72 hours of the treatment with mice showing signs of cold and inactivity. More severe cases included the death of some mice, which although is not clear at this stage, this might be the direct result of the loss of fat thus confirming that fat is important for life or an indirect effect of the toxic metabolites of CB1954. Previous experiments using transgenic technology failed to generate transgenic mice devoid of fat throughout development, which suggested that WAT was essential for life (Ross *et al.* 1993; Wang *et al.* 1995; Burant *et al.* 1997). Nevertheless, the work of Moitra *et al* (1998) and Gavrilova *et al.* (1998), confirmed the survival of mice devoid of WAT, although these two teams observed mortality of 60% and 20%, respectively, which corroborates the importance of adipose tissue for normal development of life.

The morbidity shown only by transgenic mice after treatment with the prodrug resembles a torpor-like state. Torpor, corresponds to a hibernation-like state that is characterised by inactivity, a lowered body temperature ($T_b$), and a low metabolic rate (Webb *et al.* 1982). Although metabolic rate was not measured in these mice, they did show inactivity and a low body temperature. Furthermore, histological examination of the brown adipose tissue confirmed some atrophy of this tissue thus, suggesting that non-shivering thermogenesis may have been impaired. The magnitude of the ablation correlated directly with this effect. Thus, females that received the higher dose of 40 mg/kg of body weight showed extensive depletion of the adipose stores, which was also correlated with a lower body temperature. This effect was not observed when a lower dose of 20 mg/kg of body weight was used although the ablation in these mice was not as marked as the former group.

It has been suggested that some birds and small mammals routinely use daily torpor to conserve energy, although the drop in $T_b$ may be only a few degrees. In contrast, hibernation corresponds to a state of deep torpor that can last for days to even months. Although mice and other small mammals do not use hibernation, possibly because they can not store enough fuel, it has been documented that mice can enter torpor when there is a quiet environment, reduced food availability, and a low ambient temperature (Webb *et al.* 1982).

The *ob/ob* mice are an exception, entering deep torpor upon fasting and occasionally entering torpor when well fed and housed at room temperature (Himms-Hagen, J. 1985). It has been recently reported that leptin treatment not only prevents deep torpor in these mice, but also the modest 4°C fall in the body temperature, thus suggesting that leptin has an important role in regulating $T_b$ and torpor (Gravilova *et al.* 1999). The same team also demonstrated that another mechanism (leptin-independent) is responsible for entry into torpor in a different mouse model (A-ZIP/F1 mice). This mouse model is characterised for an almost complete lack of adipose stores and undetectable levels of leptin. Interestingly, leptin treatment did not prevent these mice from entering into torpor. Thus, Gravilova *et al.* (1999), suggested that this second mechanism (leptin-independent) might be provided by a signal(s) from adipose tissue besides leptin.

In the light of these results it might be suggested that transgenic mice treated with CB1954 could enter to a torpor-like state judging by the reduced body temperature and inactivity that was observed, with this effect being the direct result of a decreased in the levels of leptin (see section 6.3 in chapter 6) or a secondary effect of the loss of fat, as was shown above. Non-transgenic mice injected with CB1954 showed no such phenotype as described with the transgenic group, nor was a drop in the body temperature observed in these mice, which suggests that the effect seen in transgenic mice treated with the prodrug is a direct effect of the activation of CB1954 with the end result being the ablation of the adipocytes.

Experiments described with females that received a dose of 40 mg/kg of body weight confirmed a reduction in the wet weight of the spleens when compared to the non-transgenic group also injected with the prodrug (Table 5.2 above). Therefore, it might be suggested that some of this variability observed in response to the prodrug is due to ectopic expression in tissues other than the adipose. In fact, section 4.6.3 in chapter 4 suggested ectopic expression in the spleen, with the possibility of ectopic expression in other tissues that were not included in that analysis. In any case, it is more likely that the ectopic expression observed in the spleen is the result of the integration site of the transgene, as the regulatory sequences used to direct the expression of NTR have been previously used by other

groups demonstrating tissue-specific expression to the adipocytes (Ross *et al.* 1990; Ross *et al.* 1992; Moitra *et al.* 1998, Gravilova *et al.* 1998).

Another potential effect of the prodrug is the possibility of a bystander effect of the toxic metabolite. As was confirmed earlier in chapter 3, NTR/CB1954 mediates a bystander effect in cells through the released of a toxic metabolite that can migrate and kill neighbouring cells. It must be underlined the fact that this is the first time a transgenic model has been created expressing NTR in different deposits of the body, as other studies have localised expression to a particular area or tissue. Thus, if produced, one might expect that this toxic metabolite diffuses to the bloodstream with the potential of causing some toxic effects elsewhere. One way to confirm this could be measuring the toxic metabolite directly in the blood of transgenic mice after treatment with CB1954.

## 5.3 CONCLUSION.

Treatment of transgenic mice with the prodrug CB1954 resulted in the selective ablation of the adipose cells whereas other tissues were normal and comparable with non-transgenic treated or untreated control mice, confirming the efficacy of this system to kill terminally differentiated (non-dividing) cells in an animal model.

Histological analysis of paraffin-embedded tissues stained with hematoxylin and eosin provided direct evidence of the change in tissue morphology. Furthermore, this analysis indicated that ablation of adipocytes was not restricted to specific adipose depots, as all major adipose depots analysed showed reduction or disappearance of the adipose cells.

The magnitude of cell ablation was correlated to the dose of CB1954 used with a much stronger effect at doses of 40 mg/ kg of body weight. This higher dose produced complete disappearance of any visible adipose depot in some transgenic mice, thus suggesting that a bystander effect may have occurred *in vivo*, since immunohistochemical analysis in sections of adipose tissue confirmed variegated transgene expression in these cells. The lack of adipocytes, at this higher dose of CB1954, had also dramatic consequences in some transgenic mice especially in their

ability to thermoregulate the body temperature. More extreme cases included the death of some mice indicating that fat is an important organ for life.

A lower dose of 20 mg/kg of body weight was also shown to be effective in ablating adipose cells of transgenic mice. Although the extent of the ablation was not as marked as with higher doses, as seen with females, these mice responded better and could be kept alive until the end of the proposed experiment, thus enabling the use of this model for studies related to the consequences of fat ablation and fatty acid metabolism. Importantly, toxic effect were not observed in the spleen of these mice although liver and kidneys were found slightly increase and decrease respectively, which might suggest an effect of the prodrug and its metabolites in these two likely routes of excretion.

# 6 CONSEQUENCES OF ADIPOSE TISSUE ABLATION USING NTR/CB1954 SYSTEM.

## 6.1 BACKGROUND.

The last chapter described the use of this novel approach NTR/CB1954 system to remove adipocytes in an animal model. Upon prodrug treatment, depletion of adipose cells in transgenic mice was observed whereas other tissues were normal and comparable with non-transgenic treated or untreated control mice. While higher doses of CB1954 produced a marked reduction in the adipose tissue of transgenic mice, these mice showed a deterioration of the condition possibly by an impaired ability to thermoregulate body temperature. This led to the premature killing of most of the experimental animals. When lower doses of CB1954 were used, a smaller effect in terms of ablation of the adipose tissue was observed, although these mice responded better and could be kept alive until the end of the proposed experiments, thus enabling the use of this model for studies related to the consequences of adipose tissue ablation.

This chapter describes some of the physiological and metabolic consequences of the ablation of adipose tissue in mice treated with the prodrug CB1954 and describes the experiments carried out to assess whether regeneration of adipose tissue occurs after ablation with CB1954.

## 6.2 DOES THE FAT REGENERATE AFTER TREATMENT WITH CB1954.

Previous experiments have shown that treatment with CB1954 lead invariably to a reduction in the body weight in a dose dependent fashion. Thus, mice injected with 40 mg/kg of body weight showed a reduction of approximately 15% and mice injected with 20 mg/kg of body weight showed a reduction of approximately 8%. The cause of this reduction has been already explained in previous chapter. Interestingly, withdrawal of the injections led to the recovery in

the body weight with this effect being more pronounced in the transgenic treated group (Figures 5.2 A and B in previous chapter and Figure 6.1 later). Thus, the question arose as whether the gain in body weight is in parallel to a gain (regeneration) of the adipose tissue lost after ablation with CB1954.

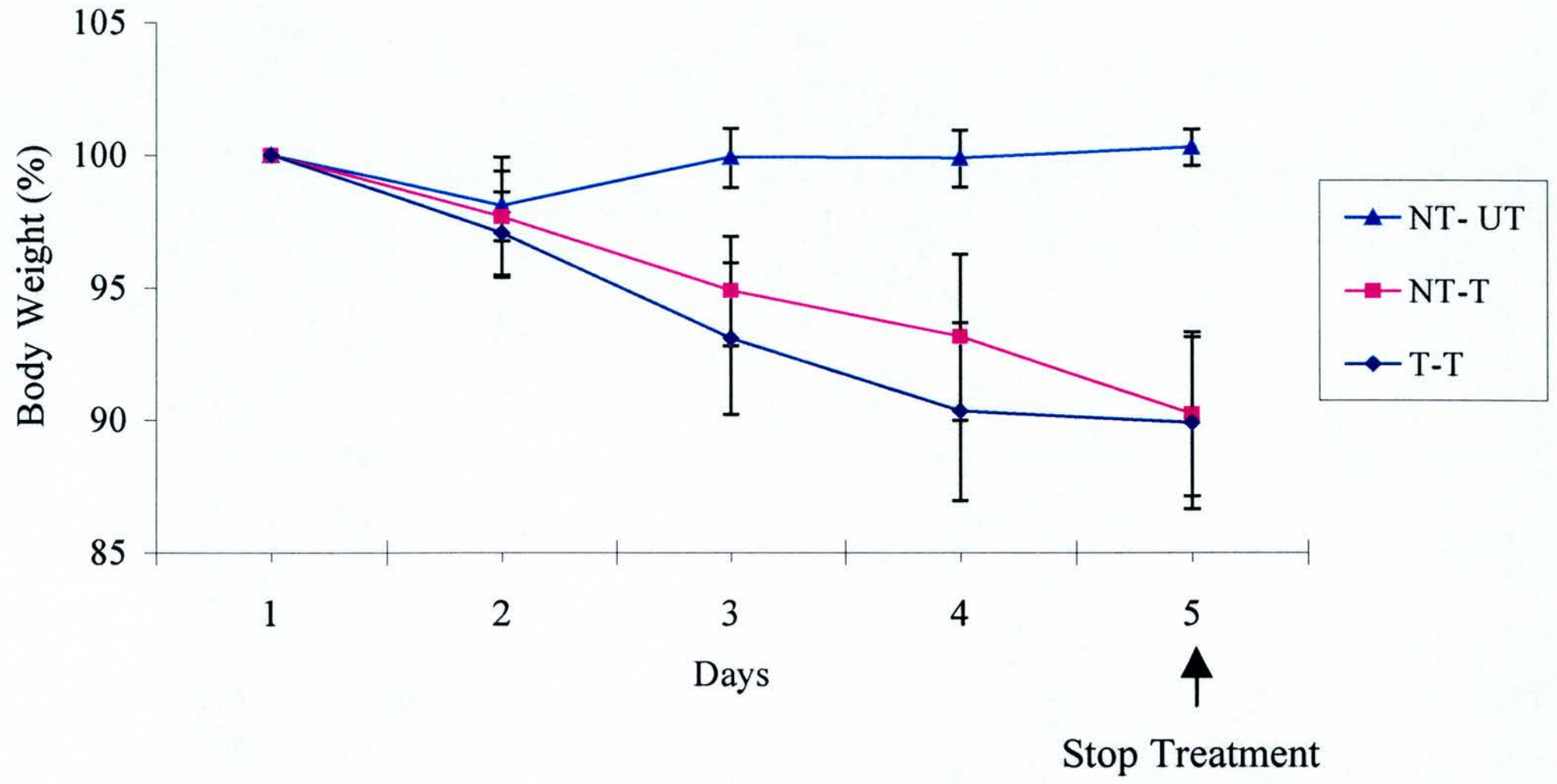
Two different approaches were designed to address this question. A direct measurement of the wet weight of some of the adipose stores after a short ablation period of 5 days and after similar ablation schedule but with a prodrug free period of 30 days. The second approach used was to measure the total body lipid content in the carcasses of these mice after similar periods as described above. The next two sections describe the results obtained in these two approaches.

### 6.2.1 DIRECT MEASUREMENT OF THE WET WEIGHT OF FAT DEPOTS AS AN INDICATOR OF ADIPOSE TISSUE REGENERATION.

For this experiment two groups of male mice (7 weeks old) were injected with CB1954 at doses of 20 mg/kg of body weight but with a regimen of 5 injections instead of 3 that had been previously used. The extension of the ablation was assessed by monitoring the change in wet weight of the epididymal fat pad (as a percentage of body weight) as these traits have been shown previously to be highly correlated to total body fat percentage in mice (Rogers and Webb, 1980). Two different groups of mice were injected with CB1954 for 5 days and sacrificed after 5 days (first group) and after a recovery period of 30 days (second group). Weight of the mice was monitored as described before starting on injection day until the mice were sacrificed.

With this regimen, 6 out of 20 transgenic mice (2 in the first group and 4 in the second group) showed loss of condition (mice hunched) and were sacrificed earlier. These mice were not included in the final analysis. Non-transgenic mice injected with the prodrug were not affected. Figures 6.1 A and B show first the effect in the total body weight of the mice after treatment with the prodrug. Transgenic and non-transgenic mice injected with the prodrug lost on average 10%

A)

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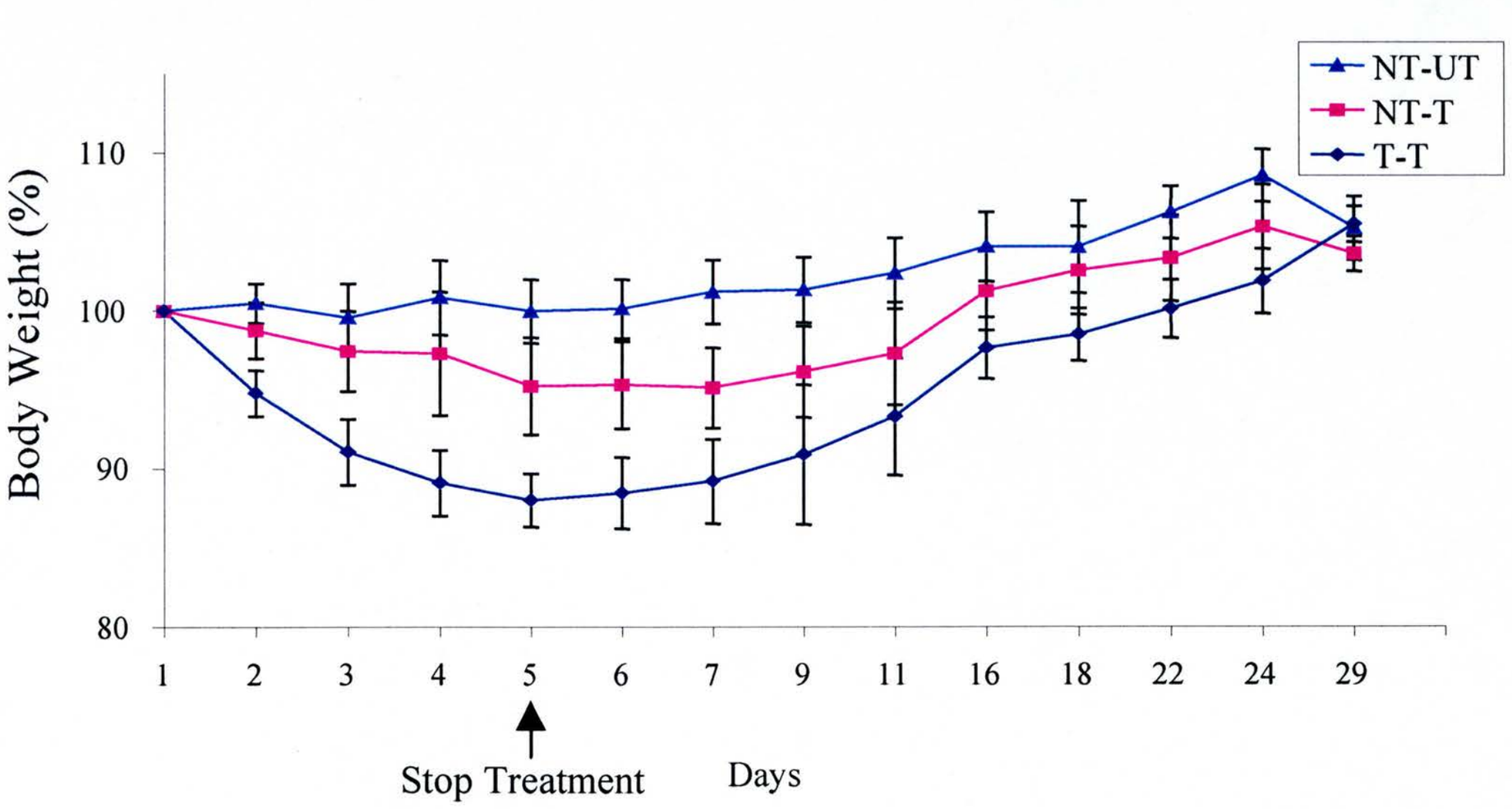



**Figure 6.1 Measurement of body weight during treatment with CB1954.**
Groups of transgenic (T) and non-transgenic (NT) mice at the age of 7 weeks were injected with the prodrug and sacrificed at day 5 (A) or after a 30 days recover period (B). The mean body weights ± SD are plotted.

of body weight in the group sacrificed at day 5 (Figure 6.1 A). In the second group, the loss in body weight was more pronounced in the transgenic group with a drop of approximately 12% compared to non-transgenic group that lost on average 4%. Withdrawal of the prodrug led to the recovery in the body weight of both groups. Interestingly, despite having lost more weight, transgenic group recovered their body weight and eventually weighed more toward the end of the experiment (Figure 6.1 B).

Comparison of the different adipose deposits and organ weights showed a significant reduction of the adipose stores especially in the epididymal fat pads of transgenic mice (Table 6.1 and Figure 6.2) that weighed on average 40% less than the non-transgenic treated group (P=0.02). A smaller effect was observed in the retroperitoneal fat pads, presumably due to the lower levels of NTR expression that has been observed in this tissue (section 4.6.4 in chapter 4), and/or due to inaccurate measurement of this small deposit. No major differences were observed with the other organs with the exception of the liver that increased significantly (P=0.002) in the transgenic group compared to the non-transgenic group. The experiment described already in previous section shows a similar effect in the liver of transgenic mice that had received 3 injection of CB1954, although the effect was not as marked as in this group.

Table 6.1 Body composition after 5 days of prodrug treatment.

| | Body Weight Grams | Epididymal (WAT) (%) | Retro (WAT) (%) | Interscap. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| T-T (n=8) | 24.7±0.8 | 0.40±0.06 * | 0.08±0.01 | 0.19±0.01 | 4.61±0.1 Ψ | 0.74±0.04 | 0.17±0.01 | 0.53±0.02 |
| NT-T (n=5) | 25.8±0.8 | 0.69±0.1 | 0.10±0.02 | 0.17±0.01 | 4.00±0.1 | 0.76±0.04 | 0.19±0.01 | 0.54±0.03 |
| P Value | | *=0.02 | | | Ψ=0.002 | | | |

Table 6.2 Body composition after a recover period of 30 days.

| | Body Weight Grams | Epididymal (WAT) (%) | Retro (WAT) (%) | Interscap. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| T-T (n=6) | 30.2±1.1 | 0.58±0.08 | 0.19±0.03 | 0.20±0.02 | 4.34±0.1 | 0.70±0.02 * | 0.26±0.02 | 0.45±0.03 |
| NT-T (n=5) | 29.5±0.8 | 0.75±0.05 | 0.21±0.02 | 0.16±0.01 | 4.36±0.1 | 0.87±0.03 | 0.21±0.02 | 0.47±0.02 |

P Value *=0.001

Data are mean ± S.E.M. Animals were transgenic and non-transgenic littermate of 10 weeks old (first group) and 14 weeks old (second group). The organ weights were measured as a percentage of the animal's body weight. Non-transgenic mice untreated were not included in this experiment due to the little number of mice available.
Probabilities (P) are from a two-tailed unpaired Student's *t* Test.

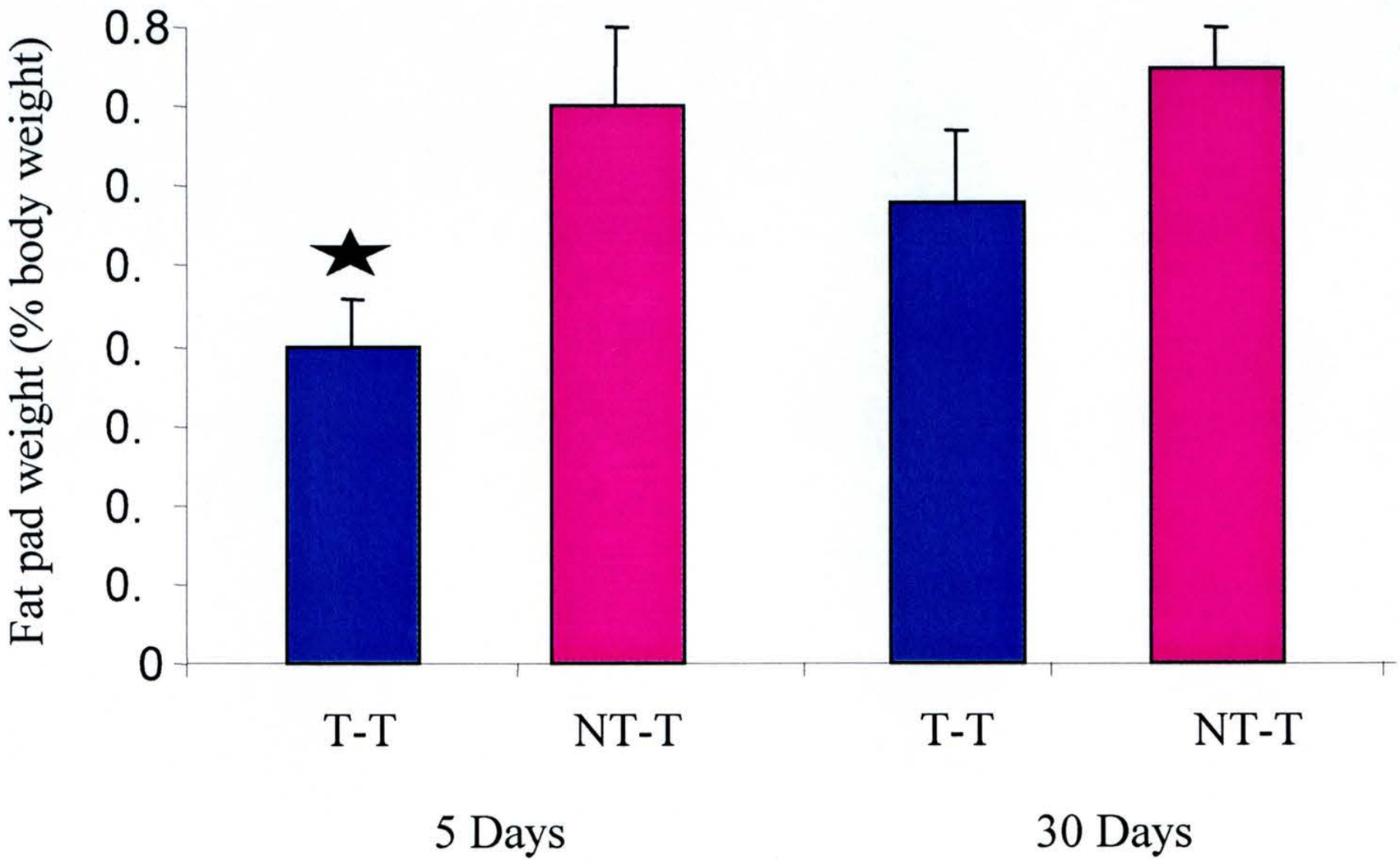



**Figure 6.2 Analysis of the fat pad weight.**
Fat pad weight was compared in transgenic (T) and non-transgenic (NT) mice treated with CB1954 at 5 days and after a recover period of 30 days, as a measured of adipose tissue regeneration.
A two-tailed unpaired Student's t Test was used for statistical analysis showing significance (P=0.02) in the transgenic group sacrificed at day 5, but not in the group sacrificed after 30 days.

After 30 days of prodrug free period, the second group of transgenic mice have increased the mass of adipose tissue, as represented by the wet weight of the epididymal (Table 6.4 and Figure 6.2) from 0.40% to 0.58% a total increased of 18%. A small increased of 8% was also observed in the non-transgenic group (from 0.69% to 0.75%). As result, the significant difference that was observed in the first group (P=0.02) disappeared in the second group (P=0.14), thus suggesting a greater degree of regeneration of the adipose tissue in the transgenic group.

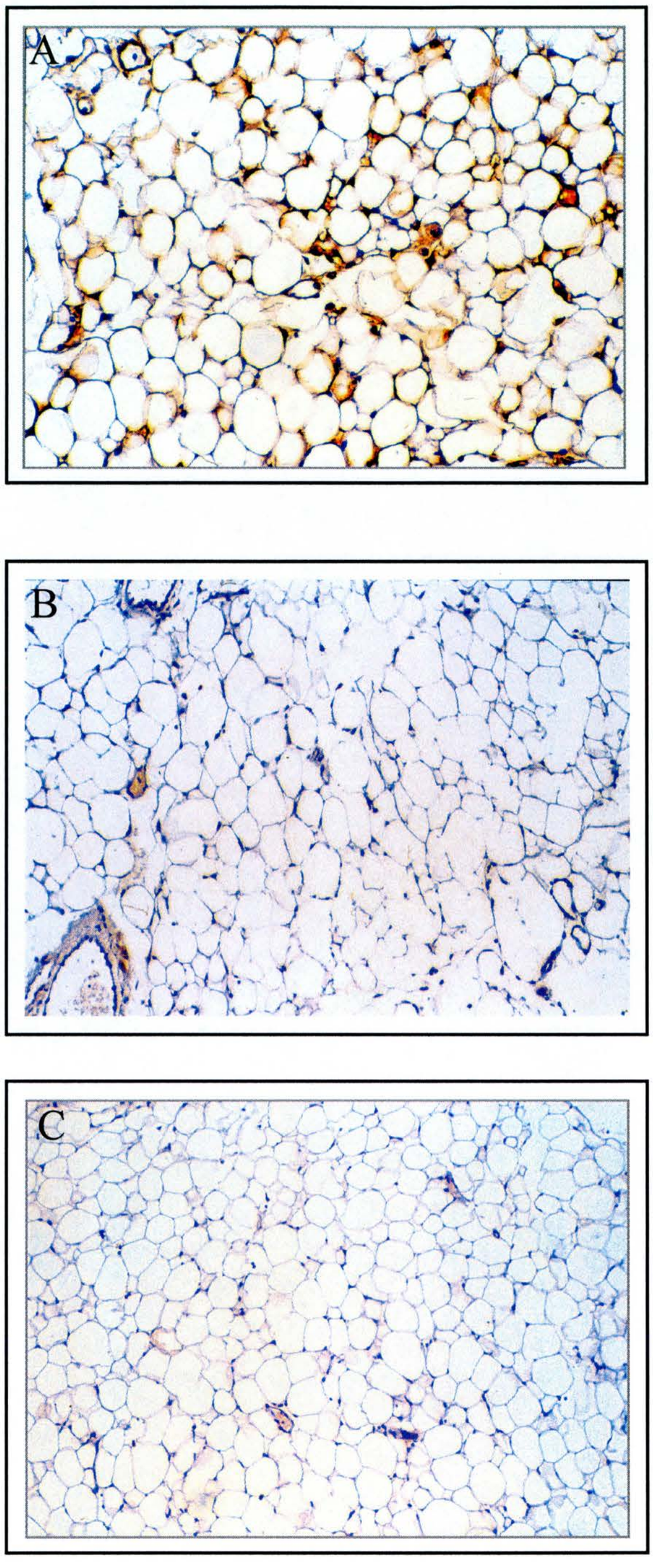
It is necessary however, to point out that the regrowth of adipose mass observed in the second transgenic group may be underestimated by the lag time at which experiments were terminated. Thus, while mice in the first group were sacrificed at day 5, the mice in the second group were sacrificed much later giving longer time to the prodrug to act and consequently for the cells to die. This might then suggest that those mice in the second group had a more marked ablation compared to the first group, which in principle would indicate that the transgenic mice in the second group have in fact recovered more fat than the data suggests in Figure 6.2. The fact that 4 transgenic mice in this group showed loss of condition and had to be sacrificed earlier confirm a stronger ablation beyond 5 days (two of these mice were sacrificed on day 7 and other 2 on day 8). Experiments carried out with cells *in vitro* (described in section 3.7 in chapter 3) showed cytotoxicity in cells stably expressing NTR that were treated with a lower dose of 100μM of the prodrug, nevertheless these cells required longer time to show similar toxicity compared to a higher dose of 200μM. A similar rationale may help us understand the stronger ablation effect that was observed in transgenic male that received a regimen of 3 injections of 20 mg/kg of body weight (as described in previous chapter). These mice showed a reduction of 50% of fat mass (as represented by the epididymal fat deposit), compared to the non-transgenic treated group, however these mice were sacrificed after 8 days of the first injection compared to the 5 days of the mice in the first group of this experiment.

In order to confirm the previous suggestion that cells may require longer time to die it was decided to carry out immunocytochemical analysis with the hope that this assay would show *in situ* the effects of the prodrug on the nitroreductase-expressing cells. Figure 6.3 A, shows the results of this assay in sections of adipose

tissue (epididymal fat pads) of transgenic mice that were killed after 5 days of treatment with the prodrug. This Figure confirms that after 5 days of treatment there is a small number of NTR-expressing cells still in the section. It is also possible to see immunostaining between the cells, which would indicate that these cells are dying or disappearing from the tissue (presumably these cells are being removed by the phagocytic machinery). Another interesting observation from this section is the large number of NTR-nonexpressing cells thus confirming the variegated transgene expression previously shown in section 4.6.5 (chapter 4). Thus, this result confirms the previous observation that 5 days may not be enough time to kill all NTR-expressing cells. On the other hand, the pattern of variegated transgene expression that is observed in these sections might also help to explain the variation that has been found, in terms of ablation of adipose tissue, in some of the transgenic mice.

After 30 days of prodrug free period, immunostaining of sections of transgenic mice have disappeared which suggest that all NTR-expressing cells have been killed. (Figure 6.3 B). Interestingly, this result indicates that NTR-expressing cells do not have the ability to regenerate after ablation. At first impression this might suggest that regeneration of the adipose tissue might be accomplished mainly by hypertrophy of remaining NTR-nonexpressing cells. Nevertheless, after a closer examination of these sections it is evident that there is a large variation in the size of the cells, with some small (immature) adipocytes growing alongside the mature adipocytes. Because the age of these mice is approximately 14 weeks, one should expect to see a very regular appearance, in terms of the size of the cells, in these sections. This might suggest then that hyperplasia (increase in the number) is also involved, and more intriguingly this hyperplasia did not include newly formed transgenic cells.

It is not clear at this point why NTR-expressing cells do not have the ability to repopulate after ablation with CB1954. One possibility is that stem cells are more sensitive to CB1954 itself. Another possibility is that ablation of NTR-expressing cells may have killed stem cells or preadipocytes by a bystander effect as observed *in vitro* (section 3.11 in chapter 3). Furthermore, this bystander effect could be enhanced by toxic cellular debris that is generated from dying cells.




CB1954 5 days
Sacrificed at day 5

CB1954 5 days
Sacrificed at day 30

CB1954 5 days
Sacrificed at day 30

**Figure 6.3 *In situ* killing of NTR expressing cells after prodrug treatment.**
Immunohistochemistry of sections of adipose tissue from transgenic mice treated and sacrificed at day 5 (A) and treated and sacrificed at day 30 (B).
Paraffin-embedded sections were hybridised with anti serum to NTR. Magnification: 200X A and B, 100X C.

Due to the developmental expression of the aP2-driven NTR transgene, direct ablation of preadipocytes would not be expected under these conditions (aP2 is expressed only in differentiated adipocytes). Further experiments are required to confirm whether these NTR-expressing cells can repopulate after a longer time than used in these experiments.

Analysis of the other organs showed that the kidneys of transgenic mice were significantly decreased in size after this 30 days recovery period (Table 6.2). This effect in the kidney might be attributed to the excretion of CB1954 itself and the toxic metabolites of this, after activation with CB1954 in the adipose tissue of NTR-expressing cells. In fact, earlier experiments carried out by Cobb, (1970) using CB1954 at toxic doses, showed similar effects in the urinary tract of rats with an increased frequency of micturition especially in females. Other effects observed by this author were histopathological changes in the liver and lung. Although lungs were not examined, livers were significantly increased in size in the transgenic group sacrificed at day 5. This difference disappeared in the second group (sacrificed at day 30), which may indicate the effects of the toxic metabolites of CB1954 being metabolised and excreted by the liver.

Histological examination of the livers showed some localised hypertrophy of hepatocytes and in some occasions the liver was congested with blood thus confirming the above observation. Experiments described in the previous chapter showed a similar tendency (although not statistically significant) in livers and kidneys of transgenic male mice that were treated with 3 injections of 20 mg/kg of body weight thus, confirming the indirect effects of the prodrug in these tissues.

CB1954 induced toxicity in mice (except in those tissues expressing NTR as a transgene) was not observed in a previous study using 50 mg/ kg of body weight, although in this study CB1954 was given for 3 days and the mice sacrificed shortly after the last injection (Clark *et al* 1997). However, in a different study mice receiving 20 mg/ kg of body weight of CB1954 for 5 days had 15% weight loss and mice receiving 50 mg/ kg of body weight experienced severe multiorgan toxicity (Drabeck *et al.* 1997). In a more recently performed study Westphal *et al.* (2000), observed hair loss using CB1954 dose of 20 mg/ kg of body weight in SCID mice, which indicates that certain mammalian cells may be able to metabolise CB1954

into a toxic form or may be more susceptible to the prodrug itself even if it remains unmodified.

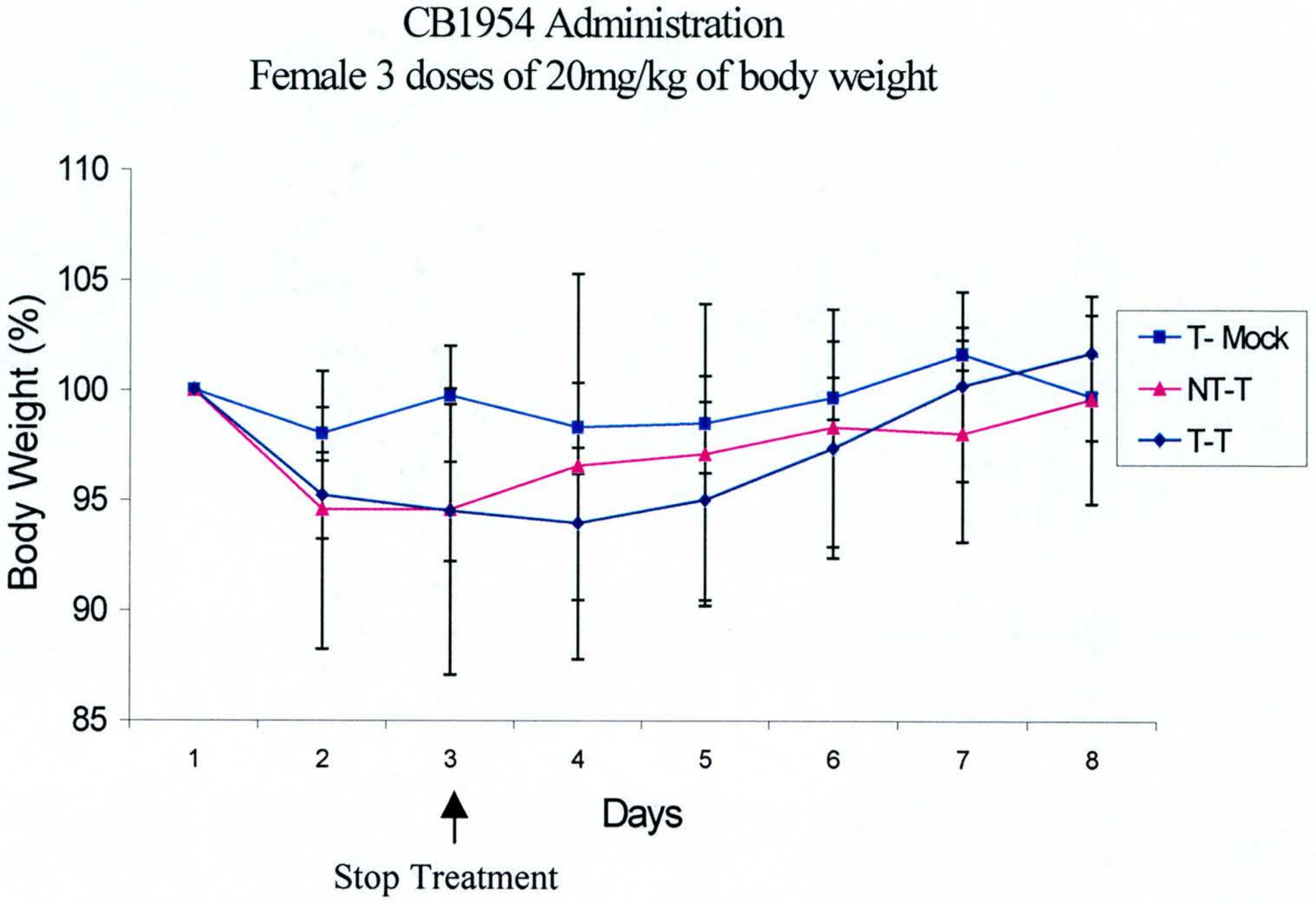
### 6.2.2 ANALYSIS OF THE TOTAL BODY LIPID CONTENT (CARCASS).

Due to the possibility of variegated transgene expression that was observed in the transgenic mice (section 4.6.5 in chapter 4), the experiments described above might have underestimated the total effect in the ablation of adipose tissue as only few selected adipose deposits were used for the analysis. To overcome this effect, it was decided to carry out an analysis of the total body lipid content with the rationale that this assay would not only demonstrate the overall reduction in the adipose tissue but also confirm whether regrowth of this tissue occurs after a recovery period without the prodrug.

Bearing in mind the loss of condition observed with doses of 40 mg/kg of body weight and with 20 mg/kg of body weight in the regimen already described of 5 injections, it was decided to use a regimen of only 3 injections of 20 mg/kg of body weight. Thus, two groups of female mice were treated with the prodrug and sacrificed at day 7 (first group) and after 30 days of prodrug free period (second group). Carcasses of these mice were frozen and sent to Roslin Nutrition for the analysis of the total body lipid content, which was carried out as described in chapter 2.

The mice used for this experiment included transgenic mice injected with CB1954, transgenic mice injected only with the vehicle solution (arachis oil), and non-transgenic mice injected with CB1954. Figures 6.4 A and B show similar effects of the prodrug in the body weight as in the previous experiment, although a less marked reduction was observed with this regimen. Transgenic and non-transgenic mice treated with the prodrug lost approximately 5-6%, respectively and withdrawal of the prodrug led to their recovery as described and discussed before.

A)




B)

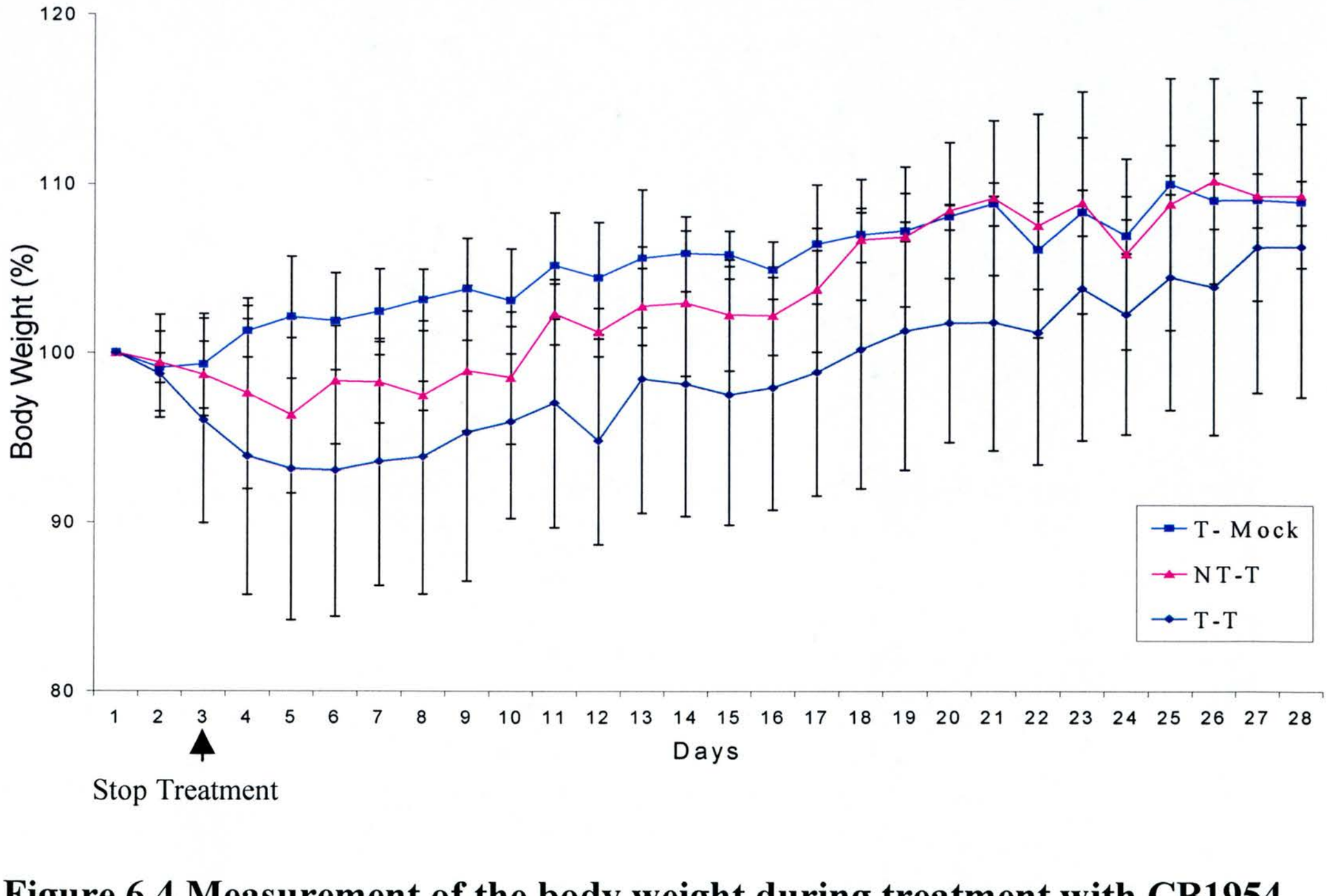



**Figure 6.4 Measurement of the body weight during treatment with CB1954.**
Transgenic (T) and non-transgenic (NT) mice were injected with the prodrug and sacrificed at day 7 (A) or after a 30 days recover period (B). T-Mock represents mice injected with the solvent solution. The mean body weights ± SD are plotted.

The results of the carcass analysis carried out in these mice allowed to confirm an effect of a lower dose of CB1954, in terms of ablation of adipose tissue (Table 6.3). Absolute fat mass was decreased in the transgenic treated group by 30% compared to the other two groups. This reduction of fat mass was significant to 94% (P=0.06) when fat was expressed as relative to body weight. As expected, relative lean body mass increased in this group. Interestingly, a second group of mice that was treated in a similar way but left to recover for 30 days showed no such difference. As shown in table 6.4, transgenic mice after a 30 days period have normalised all parameters and the significant differences that were observed in the first group are not evident any more in the second group. Thus, taken together these results suggest that after ablation of adipose tissue remaining cells are able to regenerate. The mechanism on how this happens is not clear at this point but it might involve hyperthropy or hyperplasia of the remaining cells in the tissue, as was described in previous section.

Table 6.3 Body lipid content analysis (7 days).

| | Transg. CB1954 | Transg. Mock | Non-T CB1954 | P Value |
|---|---|---|---|---|
| Body weight (g) | 18.5±1.0 | 21.0±1.0 | 21.2±1.2 | 0.22 |
| Lean Body mass (g) | 17.3±1.0 | 19.2±0.9 | 19.5±0.9 | 0.30 |
| Lean body mass % BW | 93.5±0.2 | 91.6±0.2 | 91.8±0.6 | 0.06 |
| Fat mass (g) | 1.2±0.1 | 1.8±0.1 | 1.8±0.2 | 0.06 |
| Fat mass % of BW | 6.4±0.2 | 8.4±0.2 | 8.2±0.6 | 0.06 |

Table 6.4 Body lipid content analysis (28 days).

| | Transg. CB1954 | Transg. Mock | Non-T CB1954 | P Value |
|---|---|---|---|---|
| Body weight (g) | 23.2±0.7 | 23.2±0.9 | 23.3±0.7 | 0.99 |
| Lean Body mass (g) | 20.4±0.5 | 20.7±0.8 | 20.6±0.6 | 0.96 |
| Lean body mass % BW | 88.2±0.8 | 89.0±1.0 | 88.1±0.2 | 0.72 |
| Fat mass (g) | 2.7±0.2 | 2.5±0.2 | 2.8±0.1 | 0.75 |
| Fat mass % of BW | 11.8±0.8 | 11.0±1.0 | 11.9±0.2 | 0.72 |

Values are means ± SEM; n=3.
Probabilities (P) are from ANOVA one-way.

## 6.3 LEPTIN LEVELS DECREASE AFTER ABLATION OF ADIPOCYTES.

Leptin is a hormone secreted by both white and brown adipose tissue cells in proportion to fat mass. It regulates energy homeostasis by decreasing food intake and increasing activity, sympathetic tone, energy expenditure, and insulin sensitivity (Flier, J.S 1997).

It was suggested in a previous chapter that the greater recovery in the body weight of transgenic mice might have its cause in leptin levels falling down in parallel to adipose stores after ablation with CB1954. To address the question of whether leptin levels decrease during ablation of adipose cells and more importantly if regeneration of adipose tissue, as suggested from previous sections, leads to an increase in leptin levels, it was decided to measure the plasma leptin levels in these mice after ablation and after this prodrug free period of 30 days.

Circulating leptin levels were measured with a monoclonal anti-mouse leptin radioimmunoassay by the use of the Mouse Leptin RIA Kit (Linco Research, St. Charles, MO). Individual and pooled samples of plasma were used from transgenic and control mice from the experiment described in section 6.2. It was found that after this regimen of 5 injections, levels of leptin in plasma decreased significantly by 55% in the transgenic group and slightly by 28% in the non-transgenic group compared to a control age matched untreated group (Figure 6.5). This decreased in the plasma leptin levels concomitant with the decrease in adipose stores corroborates the proposed role of leptin as an adiposity indicator (Zhang *et al.* 1994). Non-transgenic mice injected with the prodrug also showed a small reduction. The reduction observed with this group might account for the small reduction in fat weight that has also been observed with this group when treated with the prodrug.

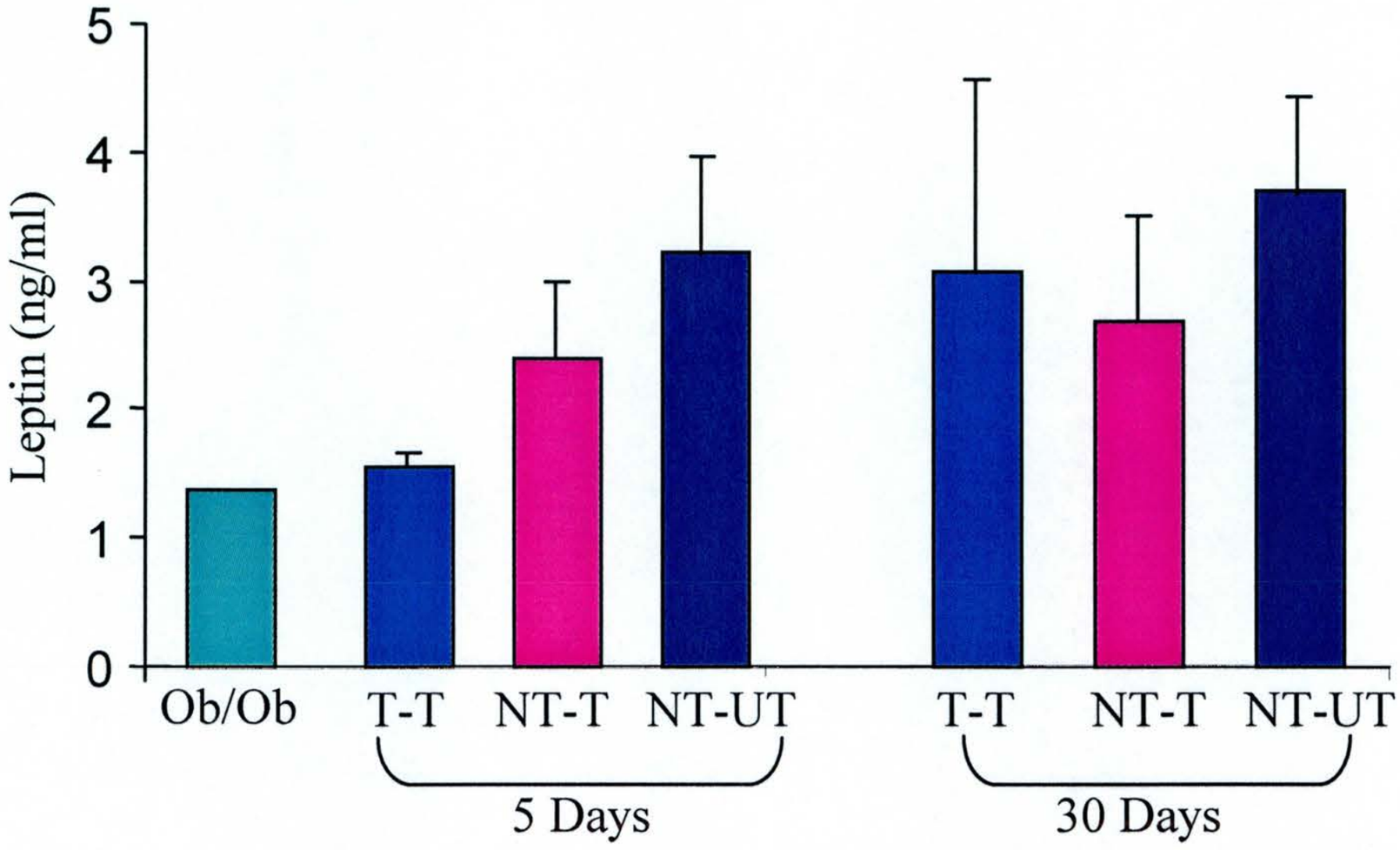



**Figure 6.5 Leptin analysis.**
Plasma leptin levels were measured in transgenic (T) and non-transgenic (NT) mice at 5 days and after a recover period of 30 days. Non transgenic mice untreated serve as control for basal levels.

Interestingly, when mice were treated as above but left to recover for approximately 30 days (second group of mice) plasma leptin levels markedly increased to levels comparable to the control group, although large variation was observed between individuals of the transgenic group. In contrast to the first group, analysis of this second group was carried out with plasma from each individual, thus the large variation observed represents the effects of each individual in this group. Although not clear, this variation between some mice in this group, one might speculate that this effect is due in part to variegated transgene expression (as demonstrated in chapter 4). Thus, for those mice with more penetrance of the transgene, ablation with CB1954 would be more effective and the possibility of a

bystander effect that would kill adipoblast or precursor cells would be enhanced. As expected, regeneration of the adipose tissue in this case would depend very much on the extension of the ablation.

It would also be interesting to observe the response of transgenic females that received a dose of 40 mg/kg of body weight (some mice showing complete disappearance of adipose tissue) over the recovery period of 30 days. One might expect from the above that these mice would not recover adipose tissue, the consequences from which could be as dramatic as those observed in the lipoatrophic models described by Moitra *et al* (1998) and Gavrilova *et al.* (1998), where the lack of adipose tissue led to diabetes in these mice. Although interesting to test, this experiment would require to have the mice in a warmer room and/or the use of heated pads continuously for 24 hours something that was not possible to carry out in previous experiments.

The results described in the last three sections strongly suggest that after ablation of the adipose tissue, remaining cells have the ability to regenerate or repopulate the tissue ablated. Tissue regeneration following toxigene ablation has been previously observed in mice and possible mechanisms have been discussed (Bernstein and Breitman, 1989). Lowel *et al.* (1993) described the generation of two transgenic mice with primary deficiency of brown adipose tissue. They used regulatory sequences of the uncoupling protein (UCP) to direct the expression of both DT-A and an attenuated form (176), as described in chapter 1, to generate mice with deficiencies in the brown adipose tissue. What these authors observed was that at an early age both lines of mice have markedly reduced brown fat and increased total body lipid content. Surprisingly, while UCP-DTA line developed marked obesity with age, in UCP-176 line brown adipose tissue regenerates later in life and total body lipid content returned to normal. A similar regeneration effect was observed in a different approach, more relevant to this thesis. Drabek *et al.* (1997) directed the expression of nitroreductase specifically in T cells using control elements of the CD2 locus. After CB1954 treatment they observed specific cell ablation in thymus and spleens. However, in a different experiment where the mice were killed after 5 days of the last injection it was observed that thymuses and spleens returned to their normal size. Therefore, the authors concluded that

progenitor cells, which did not express the transgene, are able to repopulate these tissues. In a different study where adipocytes were depleted by the use of polyclonal antibodies raised against plasma membranes of adipocytes, a compensatory increase in volume of remaining adipocytes was observed in genetically obese rats, although the same effect was not observed in diet induced obese rats (Flint, D. 1998) thus illustrating the differences between genetic- and diet-induced obesity models.

Most information regarding regeneration of adipose tissue mass comes from experiments involving direct surgical removal of adipose tissue or better known as lipectomy. Most lipectomy experiments have involved the extirpation of gonadal and/or subcutaneous-inguinal fat pads. Four possible responses have been observed to lipectomy: 1) adipose tissue may regenerate, which in the strictest sense means that adipose mass is restored at the site from which it was removed and the cells in this new tissue are newly formed, 2) there may be compensatory growth of non-excised deposits, 3) the adipose mass may diminish even further, 4) there may be no response.

Although the mechanism of regeneration observed in the adipose tissue of transgenic mice treated with CB1954 is unknown it is more likely that corresponds to hypertrophy or hyperplasia of remaining cells as suggested by the immunohistochemistry in Figure 6.3. In fact, experiments in rats that have had removed some portions of the epididymal fat pads showed regrowth after lipectomy by a mechanism of hypertrophy of remaining cells (Faust *et al.* 1977). In other experiments were the subcutaneous inguinal fat pad has been removed it has been observed regrowth of adipose tissue that is in part regenerative, with some compensatory hyperplasia of neighbouring fat (Roth *et al.* 1981).

One way to truly confirm the exact mechanism of regeneration of the adipose tissue after treatment with CB1954 involves the measuring of the tissue cellularity for which special equipment (Coulter Counter) and procedures are required which were not available at the moment that these experiments were carried out. Nevertheless, it is also important to highlight the fact that 30 days may not be enough time for this tissue to recover fully, and experiments should be considered extending this time substantially to measure the longer term effects of the ablation of the adipose tissue.

## 6.4 PHYSIOLOGICAL ANALYSIS OF MICE AFTER CB1954 MEDIATED ABLATION.

The physiological consequences of adipose tissue ablation might be anticipated from the work of clinical scientists who have studied lipodystrophic patients, a condition characterised by a marked reduction of the adipose tissue. Humans suffering from congenital or acquired lipodistrophy become severely diabetic, have voracious appetites, are hypermetabolic, and display an anabolic syndrome that includes enlargement of the liver, spleen, pancreas, and kidney (Foster D.W. 1994). Recently, two mice models have been created that are devoid of fat (Moitra *et al.* 1998; Shimomura *et al.* 1998). These models resemble many of the features observed in lipodistrophic patients, thus indicating that the lack of adipose tissue may have profound physiological consequences.

In order to investigate the physiological consequences of the ablation of adipose tissue in transgenic mice treated with CB1954, a number of parameters were measured in the plasma of these mice after treatment with the prodrug. These parameters included total blood glucose levels, and plasma triglycerides, free fatty acids (FFA) and β-hydroxybutyrate levels (Figure 6.6). Pooled samples of plasma were used from transgenic and control mice from the experiment described in section 6.3.1, except for glucose that corresponds to individual determinations. After this regimen of 5 injections, blood glucose levels are preserved and remained comparable to the controls indicating no major problem in withstanding the reduction of adipose tissue. Triglycerides, which are carried in the circulation as lipoprotein particles, were slightly elevated although this difference was not significant with any of the control groups. Similarly, plasma free fatty acids (FFA), which are a circulating energy source were slightly elevated in the transgenic group however due to the high variation observed in this group this difference was not significant. One possible explanation for this slight increase might be due to start the experiments relatively late during the light cycle. Another possible explanation might be the oxidation of triglycerides from ablated adipose cells, thus indicating a possible destination for the ablated fat. β-hydroxybutyrate is a ketone produced by the liver from free fatty acids at times of low glucose, low insulin and high glucagon levels and increases in diabetes or after starvation.

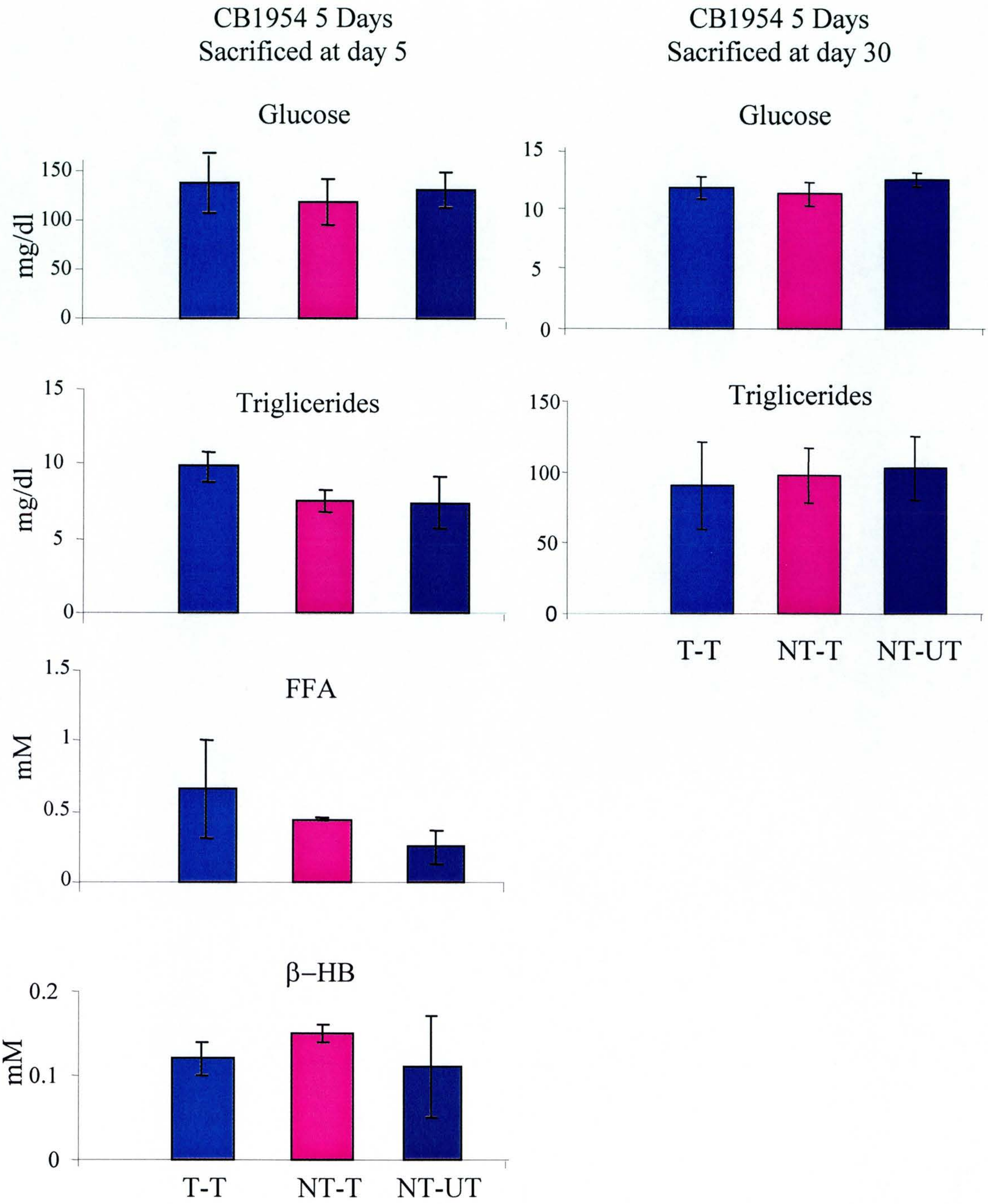



**Figure 6.6 Physiology of mice after treatment with CB1954.**
Male mice transgenic and non-transgenic control were injected with CB1954 for 5 days and sacrificed at day 5 (first group) and at day 30 (second group).
T-T: Transgenic treated, NT-T: Non-transgenic treated, NT-NT: Non-transgenic non-treated.

Plasma levels of β-hydroxybutirate were unchanged in the transgenic group compared to the controls, thus suggesting no increased production of ketones bodies by the liver.

Taken together these results suggest no major metabolic consequences of the ablation of adipose tissue at least as described in this regimen of 5 injections of 20 mg/kg of body weight, with blood glucose, triglycerides, FFA and β-hydroxybutirate levels remaining normal or at levels comparable to the control groups. This does not rule out of course the possibility that other parameters that were not included in this analysis may have been altered, such as hormones involved in fatty acids metabolism and glucose homeostasis (e.g. leptin as was shown in previous section).

After a recovery period of 30 days blood glucose levels and triglycerides remained normal, which tend to confirm the ability of the adipose tissue to regenerate in these conditions. This hypothesis is based in the fact that without an appropriate amount of adipose tissue to take up and store free fatty acids/triglycerides derived mainly from diet and hepatic production, blood levels would have become elevated. Increased levels of FFA in turn would have increased the glucose levels via the glucose fatty acid cycle, in which muscle burns FFA in preference to glucose (Randel *et al.* 1963; Gavrilova *et al.* 2000) and increased levels of triglycerides in the circulation would have finished with a marked increased in hepatic triglycerides, a condition known as steatosis, that was not observed in hematoxylin and eosin sections of livers of these mice. Nevertheless, one might speculate that a different situation would have been observed if regeneration of adipose tissue does not occur. This would be true especially in those mice that showed a dramatic ablation of the adipose tissue after they received an injection of 40 mg/kg of body weight with some mice showing complete disappearance of visible adipose tissue. In that case, some possible scenarios expected to be observed in these mice are like those described in the literature for transgenic models devoid of fat.

Burant *et al.* (1997) for instance, described a delayed-onset form of lipodistrophy that appeared when adipose tissue of a mouse was genetically ablated by targeted expression of the diphtheria toxin A (DT-A) transgene under control of

the aP2 promoter/enhancer. These mice were phenotypically normal at birth and showed no signs of lipodistrophy until 5-6 months of age, at which time the adipose tissue begun to disappear. Two other models of lipodistrophy showed very similar physiological consequences of the ablation of adipose tissue although the magnitude of adipose tissue depletion was much higher ~99% and ~70% Moitra *et al.* (1998) and Shimomura *et al.* (1998), respectively. Many of the animals died before they reached adulthood, but those that survived developed diabetes. Their cells no longer responded properly to insulin, as a result insulin levels in the bloodstream were increased up to 442 times normal levels for some mice and glucose levels at least tripled. Like human diabetics, the animals also had high levels of triglycerides and other fat building blocks in their circulation, and their organs became enlarged with the liver showing the presence of triglycerides.

## 6.5 CONCLUSION

Ablation of adipose tissue with a lower dose of CB1954 resulted in a partial reduction of this tissue leaving some remaining cells that do not express the transgene and thus escaped the ablation with the prodrug. Partially ablated adipose tissue showed the ability to regenerate after a prodrug free period of 30 days. This observation is based on the results carried out measuring the content of the total lipid in the carcasses of the mice as well as by measuring the wet weight of the fat depots. Interestingly, immunohistochemistry analysis showed that the compensatory growth of adipose tissue did not include newly formed transgenic cells. The reason for this is not clear but a possible scenario might be that preadipocytes or stem cells died as result of a bystander effect. Although the mechanism of regeneration of the adipose cells is not clear it might involve hyperplasia and/or hypertrophy of the remaining cells. This observation is based on the results of the immunohistochemistry that showed a number of small adipocytes growing alongside some much larger fat cells.

No major physiological consequences were observed from the ablation of adipose cells with a lower dose of CB1954. Blood glucose, and plasma triglycerides, FFA and β-hydroxybutirate levels remained normal or at levels comparable to the control groups after a short ablation period of 5 days. After 30 days, blood glucose levels and triglycerides were comparable to controls thus indicating no longer term effects at least for these two parameters. Nevertheless, analysis of leptin showed that this hormone decreased significantly after ablation of adipose tissue thus, corroborating the proposed role of this hormone as an adiposity indicator, the levels of which returned to normal after this recovery period of 30 days.

Inducible ablation of adipocytes in this transgenic model provides unique opportunities to study the effects of decreasing the number of adipocytes at different stages of the mouse development, allowing the investigation of the interrelationship between excessive adipose tissue mass and the disorders associated (diabetes, hypertension, hyperlipidemia) through manipulation of the target cell population.

# 7. EXPLOTING OTHER POSSIBILITIES OF THIS INDUCIBLE SYSTEM NTR/CB1954.

The present chapter describes two approaches exploiting the potential use of this inducible system NTR/CB1954. In the first approach CB1954 was tested for its ability to cross the transplacental barrier to act directly in the ablation of adipose cells (brown fat) in the fetuses. In the second approach, the *ntr* transgene was introgressed into an obese line by mating one of the transgenic lines with heterozygous $Lep^{ob}$/+ mice. These mice were then treated with CB1954 and the effects of the prodrug on adipocytes and physiology of the mice assessed.

## 7.1 IN UTERO ABLATION.

An interesting property of the NTR/CB1954 system is the ability of the prodrug to cross different cellular membranes. Such property has been already exploited by Cui *et al* (unpublished results) to show the feasibility of this system to induce ablation of astrocytes in the central nervous system, for which CB1954 had to cross the haematoenchephalic barrier. Therefore, it was envisaged that such property could be further exploited by attempting ablation experiments *in utero.* This should allow the killing of different cellular types before birth, something that is not possible to carry out with other gene suicide systems such as Diphtheria toxin or Ricin A.

Because brown adipose tissue is formed at day 15 of gestation (Houstek *et al.* 1988), it was expected that injections of CB1954 to a 14 days pregnant female could result in the specific ablation of this tissue in transgenic offspring. On the other hand, white adipose tissue should not be affected as it has been shown that its development is postnatal (Ailaud and Hauner, 1998), although it could not be ruled out the possibility that committed preadipocytes were affected. It was envisaged that these experiments could provide a new model to study the consequences of brown adipose tissue ablation. Because of the capacity of BAT to uncouple mitochondrial

respiration, it has been implicated as an important site of facultative energy expenditure. Therefore, BAT deficiency might be expected to cause reduced energy expenditure and obesity.

## 7.2 CB1954 DOSAGE.

Preliminary experiments were carried out to determine optimum levels of CB1954 for the generation of live-born pups, because results for such experiments were largely unanticipated. Transgenic males were crossed to non-transgenic females to avoid the effects of the prodrug in ablating the adipose tissue of the dam. This maternal injection protocol should result in the generation of fetuses that are transgenic and non-transgenic, the latter of which provides an internal control for the ablation effects of the prodrug in the transgenic siblings. Six F1 females were mated to transgenic male of the line 87 (group A). As control, the same number of F1 females was mated to F1 males (group B). 3 females from each group (A and B) received 5 injections of 20 mg/kg of body weight, starting on day 14 of gestation until day 18. Remaining 3 females from each group (A and B) received 5 injections of 30 mg/kg of body weight.

At doses of 30 mg/kg of body weight, all pups generated from F1 females mated to transgenic males (group A) were found dead. A similar effect was also observed with the control non-transgenic group (group B) which had only one litter that survived. On the other hand, a lower dose of 20 mg/kg of body weight allowed the live birth of 16 pups from the three dams in the group A however, PCR analysis after weaning of these mice showed that no transgenic offspring had been generated from these mice thus, indicating no survival of transgenic pups. The control group (group B) had only 11 pups generated from 2 dams as the remaining litter from the third dam in this group was found dead. These results established that CB1954 at doses of 30 mg/kg of body weight results in toxicity to either the pups or the dam's ability to nourish the offspring. Visual inspection of the dead pups showed some milky fluid in their stomachs, which might suggest that these pups were born alive but they died due to inappropriate care from the dams. Experiments with 20 mg/kg of body weight generated no-transgenic pups alive, which might indicate a direct

effect of the prodrug activating the transgene in those mice. Although is not clear whether these transgenic pups were born alive and died as consequence of the loss of brown adipose tissue. Nevertheless, the fact that 1 litter was found dead in the control group still suggests some effects in the female's ability to nourish the pups.

Therefore, a second experiment was set up this time with a regimen of only 3 injections of 20 mg/kg of body weight, starting on day 14 of gestation until day 16. Six F1 females were mated with transgenic males of the line 87. Three of these females were injected with CB1954 and the other three were left untreated to serve as a control for the number of transgenic pups generated. Three F1 females were also mated with F1 males and were injected with CB1954. With this regimen, all dams gave birth to live offspring. Nevertheless, the number of pups per litter was largely decrease for those dams mated with a transgenic male that had been treated with the prodrug, which suggested an effect of the prodrug in the transgenic offspring. Confirmation of this came after analysis of the transgenic status of these mice, which was carried out after weaning (approximately 21 days). This analysis was done as usual from tail-tip DNA as described in chapter 2. PCR analysis confirmed a significant decrease in the number of transgenic pups generated from the 3 dams that had received the prodrug. Only two transgenic pups were found positive for the transgene (13%), which is in marked contrast with the control untreated group that had 14 transgenic pups (58%). In fact, the number of pups from these 3 dams treated with the prodrug accounted for only 15, which is in contrast to the other transgenic non-injected and control F1 injected dams that had 24 and 23 pups, respectively thus indicating no lethality in these control groups (Table 7.1).

Although difficult to assess, it is possible that transgenic pups may have been born alive and then succumbed due to the deficiency of brown adipose tissue. A more direct observation of pups born from these dams should be exercised in future experiments to confirm this suggestion.

Birth weight and length of pups born from these dams showed a significant difference in both parameters ($P=<0.0001$, $P=<0.0001$, respectively) when compared to the control untreated group by the criterion of Student's *t* test. However, this effect was also observed for the control group (F1xF1) whose dam had been injected with the prodrug ($P=<0.0001$, $P=<0.0001$, respectively), which at first impression

might indicate an indirect effect of the prodrug in the normal development of the pups. Nevertheless, all dams injected with CB1954 had delivered earlier than expected (day 18 of gestation), which suggests that induction of parturition, due to stress-manipulation during the injections, may have occurred. This could explain the smaller size and weight that was observed in these pups since this effect was not observed in the control untreated group that gave birth as expected at day 21±1.

Table 7.1 Effect of the administration of CB1954 to pregnant dams on weight and length of pups at birth.

| Offspring from: | F1 x Tg (CB1954) | F1 x F1 (CB1954) | F1 x Tg (No-CB1954) |
|---|---|---|---|
| Total Births observed | 15 | 23 | 24 |
| Weight at birth (g) | 1.24±0.03 * | 1.38±0.05 * | 1.83±0.04 |
| Length at birth (cm) | 29.1±10.44 * | 29.2±0.37 * | 32.8±0.40 |

Data are mean ± S.E.M.
Each group was compared independently with the control untreated group by the criterion of Student *t* test.
*P<0.0001

The remaining two transgenic pups (males) were studied in more detail to assess the consequences of the *in utero* ablation with the prodrug in the brown adipose tissue of these mice. Body weight of these 2 pups was monitored in a weekly base together with 2 male siblings of the same litter (F1xTg CB1954) until the mice were sacrificed (~8 weeks). Also, two male pups that were born from a dam crossed to a non-transgenic male (F1xF1) and two other male pups from a dam that was crossed with a transgenic male but that was not injected (F1xTg No-CB1954) were included as control groups.

Analysis of the body weight of these mice showed a slight decrease in the size of the siblings, transgenic and non-transgenic, of approximately 10% when

compared to the control groups, with this effect being more pronounced in the two transgenic mice of this litter (Figure 7.1 and Table 7.2). The two non-transgenic mice that were born from a dam that was also treated with the prodrug (F1xF1 CB1954) showed similar body weight to the control untreated group (F1xTg No-CB1954).

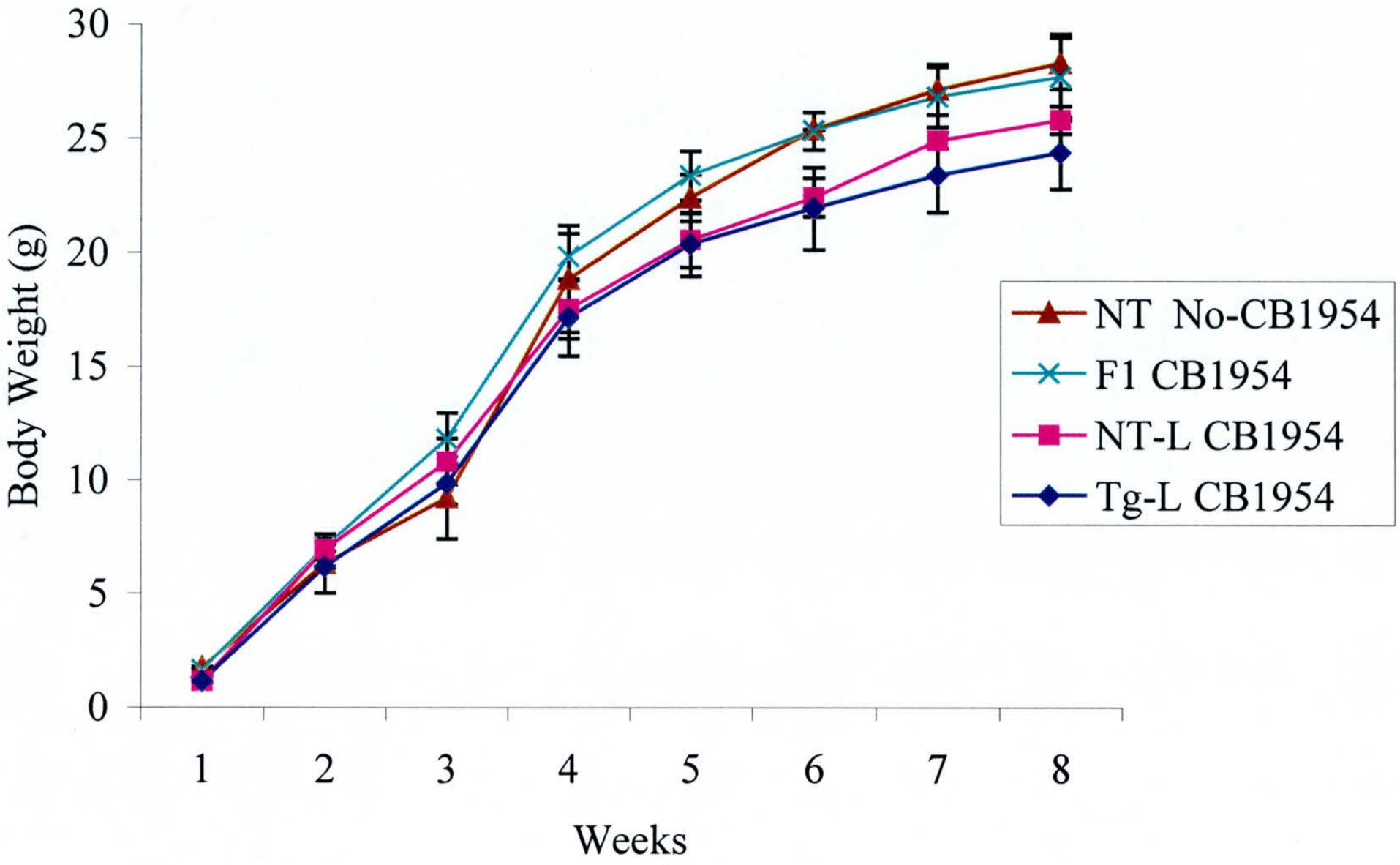



**Figure 7.1 Effects of CB1954 administration during gestation on the growth of transgenic and non-transgenic pups.**

Body weight of male pups from each of the respective groups was monitored once a week for 8 weeks.

Tg-L and NT-L are transgenic and non-transgenic mice (siblings) from the dam injected with CB1954 (F1xTg CB1954).

NT: mice from F1xTg NoCB1954 group.

F1: mice from F1xF1 CB1954 group.

Dissection of these mice after this 8 weeks period showed a large reduction in the brown adipose tissue of both transgenic mice in comparison with that one of siblings brothers and remaining mice of the other groups (Table 7.2). This was especially evident in one of the transgenic mice that had a very small adipose depot (Figure 7.2). However, when compared statistically with the other groups, there was not significant differences due to the large variation that was observed in this transgenic group and one of the control groups (P=0.09). Another difference observed in the transgenic group was a marked increased in the size of the heart (P=0.02). Although it is not clear this effect in the heart one might speculate that this effect corresponds to an homeostatic response due to secondary effects of the ablation of the brown fat, although further analysis are required to assess this hypothesis more directly. The white adipose tissue of these mice was comparable to that one of control mice thus suggesting no effect in this tissue.

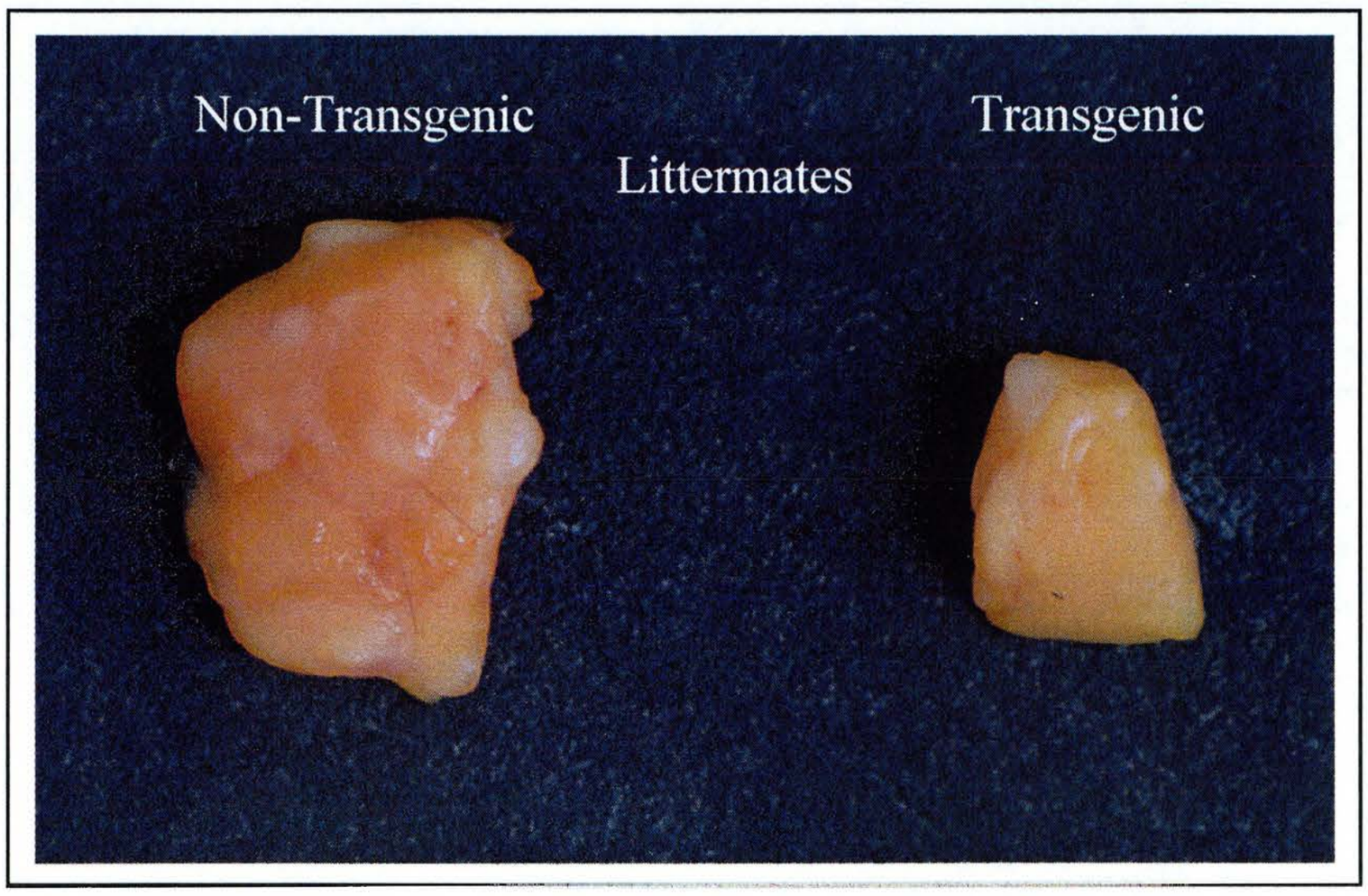



**Figure 7.2 Photomicrograph of interscapular brown adipose tissue (BAT).**
Photomicrograph of brown adipose tissue (interscapular deposit) of two brother mice whose dam was injected with CB1954 at day 14 of gestation. On the right side of the picture it is possible to observe the large decrease of this organ compared with the non-transgenic littermate.

A large decrease in the size of the testes was also observed. Intriguingly, this effect was observed in all mice whose dam had been injected with CB1954, regardless of transgenic status or genetic background (Figure 7.3). This effect was highly significant ($P<0.0001$) when all mice were compared by ANOVA (Table 7.2), which confirms the ability of CB1954 to cross the transplacental barrier. Nevertheless, this indirect effect in the testes was not anticipated, since previous experiments where CB1954 was administered via i.p. in mice (as described in chapter 5 and 6) showed no such effect, even at the higher doses of 40 mg/kg of body weight (chapter 5). It is possible that this corresponds to a longer-term effect of the prodrug, which future experiments should confirm whether this effect is observed immediately after birth of these mice or if it corresponds to a much longer residual effect of the prodrug in this organ. It is not clear from toxicology experiments carried out by Cobb, (1970) whether this effect was observed in the testis since there is no reference of it, nevertheless this author observed histopathological changes in the urinary tract, liver and lungs in experiments with CB1954 at the minimum toxic dose. A similar effect was also described, although increased in magnitude, when CB1954 was administered at maximum tolerated doses. As previously mentioned in chapter 5, these results confirm the need of further studies with CB1954 to asses the longer term effects of the treatment with this prodrug.

It would have been interesting to asses the fertility of these males simply by housing these mice with 2 or 3 females and counting the number of plugs after a couple of weeks. As well as direct measurements of testosterone levels should provide evidence to confirm whether this reduction in the size of testes correlates with a reduction of the reproductive capacity of these mice.


**Figure 7.3 Photomicrograph of testes.**
Photomicrograph of testes showing the reduction in size that was observed in all mice whose dam was treated with the prodrug.

Table 7.2 Body composition of mice born from dams treated with the prodrug.

| | Body Weight Grams | Gonadal (WAT) (%) | Retro (WAT) (%) | Intersc. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) | Testis (%) |
|---|---|---|---|---|---|---|---|---|---|
| Tg-T-L (n=2) | 24.4±1.0 | 0.83±0.15 | 0.25±0.01 | 0.11±0.03 | 4.41±0.1 | 0.73±0.14 | 0.29±0.03 | 0.64±0.01 * | 0.01±0.002 § |
| NT-T-L (n=2) | 25.8±0.5 | 1.26±0.11 | 0.35±0.03 | 0.20±0.01 | 4.21±0.1 | 0.80±0.04 | 0.22±0.02 | 0.45±0.02 | 0.02±0.005 § |
| NT-T (n=2) | 28.3±1.0 | 0.92±0.01 | 0.25±0.01 | 0.19±0.01 | 4.31±0.2 | 0.86±0.14 | 0.21±0.01 | 0.48±0.04 | 0.10±0.005 |
| F1-T (n=2) | 27.7±1.1 | 0.91±0.13 | 0.24±0.05 | 0.18±0.03 | 3.10±0.4 | 0.76±0.01 | 0.29±0.03 | 0.52±0.03 | 0.01±0.002 § |

P Value * <0.05 §<0.0001

Body composition of the two male pups from each of the respective groups at the age of 8 weeks. CB1954 was administered to the dams of T-L, NT-L and F1 groups.
Tg-L and NT-L are transgenic and non-transgenic mice (siblings) from the dam injected with CB1954 (F1xTg CB1954).
NT: mice from F1xTg NoCB1954 group.
F1: mice from F1xF1 CB1954 group.
Data are means ± S.E.M. Groups were compared by ANOVA one way.

The work of Lowell *et al.* (1993) indicated that brown adipose tissue-ablated mice become obese due to a reduced metabolic rate. It was later confirmed (Klaus *et al.* 1998) that these mice had a downshift of Tb level of 1°C when compared with the control group and therefore it was suggested that this could significantly have an effect on the overall energy budget of these mice.

A similar effect in the body temperature of these mice whose dam had been treated with CB1954 was expected to observe. However, no such effect was observed with rectal body temperature being actually slightly higher than control groups (Figure 7.4). Furthermore, histological examination of the brown adipose tissue in these mice showed no signs of atrophy, which suggests that thermogenesis, was essentially not impaired (Figure 7.5).

A main difference observed in this model compared to the model of Lowell *et al.* (1993) which might help to explain the discrepancy in the response observed to the ablation of brown fat (BAT), is precisely the appearance of this tissue. While the mice whose dam was treated with CB1954 showed small deposits of BAT, histological examination of these deposits confirmed the appearance of a normal rich-eosinophilic tissue without any signs of atrophy. In marked contrast, the BAT in the models of Lowell and coworkers was largely increased in size being almost 3 times heavier than control group with this effect being mainly due to increased lipid deposition because the protein content was not different from the controls. Furthermore, the thermogenic capacity of this tissue, as assessed by measurement of the cytochrome oxidase activity (COX), an indicator of the total respiratory capacity, was largely reduced in the transgenic mice of Lowell and coworkers being only 21% of controls and the content of UCP was reduced to 15% of control.

These observations suggest a different response to the ablation of brown adipose tissue with these two approaches. While diphtheria toxin A, as used in the model of Lowell and coworkes, produced transgenic mice with primary deficiency of brown adipose tissue, CB1954/NTR produced only a partial reduction of this tissue without, apparently, affecting its overall function.

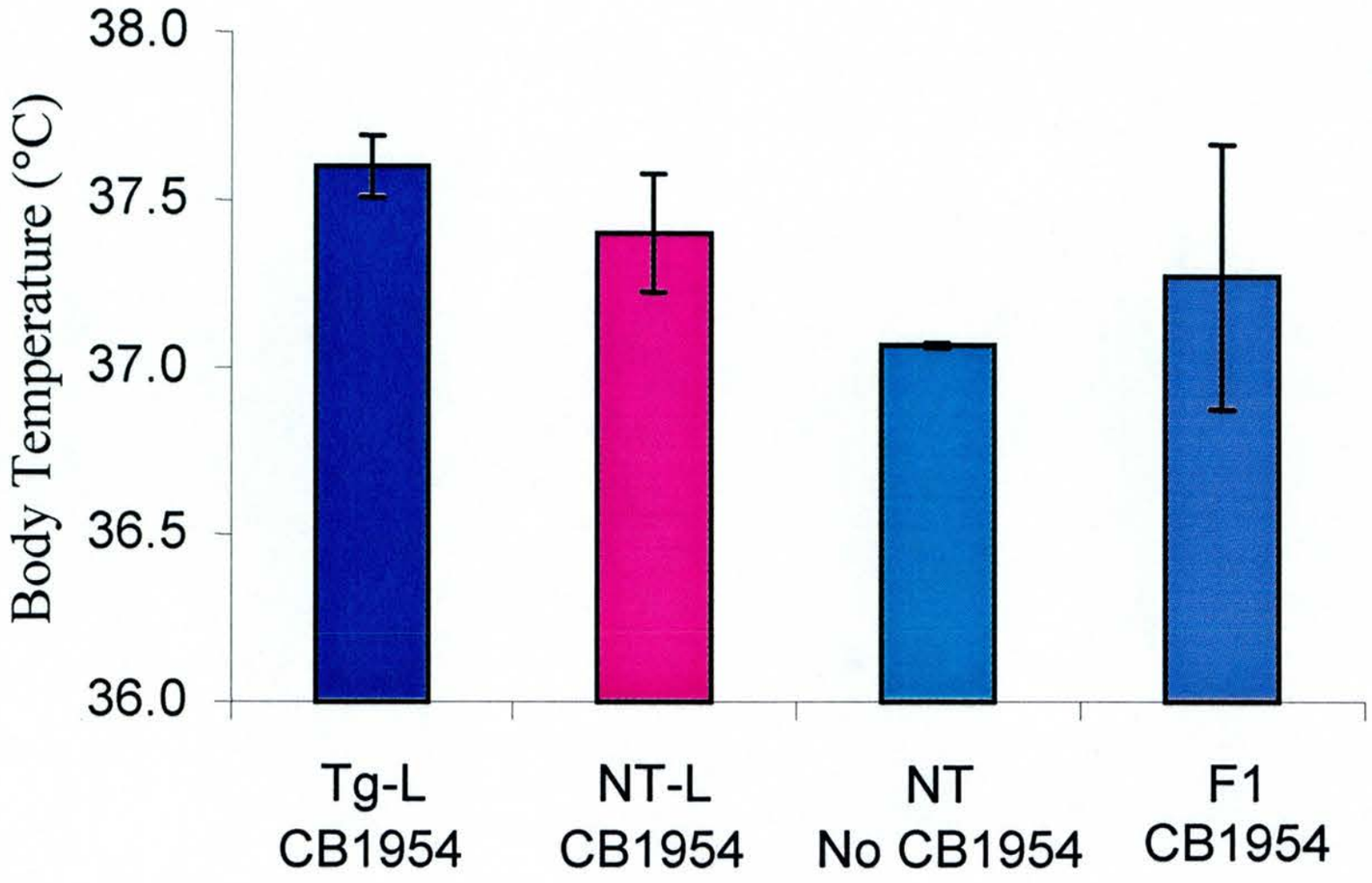



**Figure 7.4 Measurement of body temperature.**
Core body temperature was measured in the morning with a rectal thermometer.
Tg-L and NT-L are transgenic and non-transgenic mice (siblings) from the dam injected with CB1954 (F1xTg CB1954).
NT: mice from F1xTg NoCB1954 group.
F1: mice from F1xF1 CB1954 group.

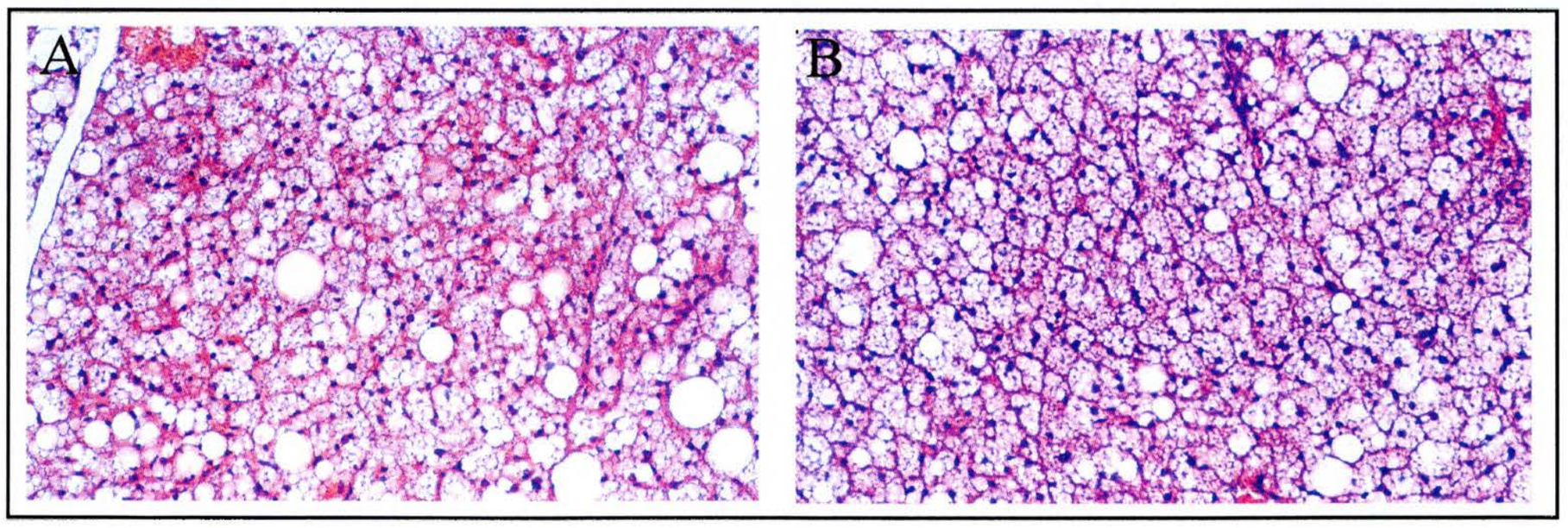



**Figure 7.5 Histology of brown adipose tissue (BAT).**
Histological sections of the brown fat of a transgenic (B) and a littermate non-transgenic mouse (A) whose dam was injected with CB1954 at day 14 of gestation. Tissues were fixed in NBF and paraffin-embedded sections stained with haematoxylin and eosin. Magnification: 200X A and B.

Nevertheless, these conclusions must be taken with caution since it is possible that a similar effect would have been observed in those transgenic mice that died possibly due to the deficiency of BAT. Therefore, future experiments should consider more detailed analysis of these mice especially to assess the magnitude of BAT ablation at birth. In this way, immunoblotting with UCP antiserum to determine the interscapular BAT content of UCP should confirm the magnitude of the BAT ablation, as well as tissue cellularity and protein content of this deposit. Thermogenic activity of this tissue could be assessed by measurement of the COX activity and basal metabolic rate (resting oxygen consumption). Also, continuous telemetric recording of core body temperature with temperature-sensitive radio transmitters should clarify whether brown fat thermogenesis is indeed increased as suggested by the slight increased in rectal body temperature and smaller size of these mice that was observed. Finally, cold acclimation experiments should also provide important information regarding the ability of the remaining brown fat to respond to cold.

Another possibility is that these mice had indeed an increased metabolic rate as result of the partial reduction in the brown adipose tissue that was observed. This would suggest that remaining brown adipose tissue would be more active, which would explain the higher body temperature and the leanness observed in these mice. This could also explain the significantly increased size of the heart in these mice. Although provocative, this theory needs to be confirmed with further studies and a much larger population of mice. Studies carried out by Cummings *et al.* (1996) confirmed that higher body temperature could increase metabolic rate in mice. These authors created genetically lean mice by targeted disruption of the RIIβ subunit of the protein kinase A. These mice are resistant to dietary induced obesity due to chronic activation of BAT thermogenesis, resulting in elevated body temperature (0.8°C) compared with the control groups and thus increased metabolic rate. In a different experiment Erickson *et al.* (1996) created *ob/ob* mice deficient for neuropetide Y. These mice showed an attenuation of their obesity syndrome partly due to enhanced energy expenditure by maintenance of a higher body temperature. Therefore, if ablation of brown fat in these mice whose dam was treated with CB1954 is producing an increase metabolic rate, experiments should be attempted to

clarify this and to assess whether these mice could be resistant to diet induced obesity as the mice of Cummings and co-workers are.

Previous attempts to ablate or denervate brown adipose tissue surgically were uninformative because it exists in diffuse deposits and has substantial capacity for regeneration and hypertrophy (Rothwell and Stock, 1989). This capacity for hypertrophy also suggests the need to analyse these mice soon after birth, because it is possible that these two transgenic mice were born with much smaller deposits of brown fat enough to allow them to survive and then this tissue had regenerated. Future experiments should consider the analysis of these fat deposits at birth to clarify this point.

Physiological studies of these mice showed no major differences in any of the parameters measured with blood sugar levels and plasma triglycerides and FFA within normal ranges for all groups (Table 7.3), although the low number of mice makes statistic analysis difficult. These results suggest that a partial reduction of the brown adipose tissue has no major physiological effect in these mice.

Table 7.3 Physiological characteristic of mice.

| | Glucose mg/dl | Triglicerides mg/dl | FFA mM |
|---|---|---|---|
| Tg-T-L (n=2) | 137±12.7 | 116±9.9 | 1.0±0.6 |
| NT-T-L (n=2) | 142±10.2 | 84±1.4 | 1.3±0.2 |
| NT (n=2) | 143±16.5 | 118±16.2 | 0.9±0.1 |
| F1 (n=2) | 160±32.4 | 98±13.4 | 1.0±0.01 |

Data are mean ± S.D. Animals were males 8 weeks old.
Tg-T-L and NT-T-L are transgenic and non-transgenic mice (siblings) from the dam injected with CB1954 (F1xTg CB1954).
NT: mice from F1xTg NoCB1954 group.
F1: mice from F1xF1 CB1954 group.

It was anticipated from previous work studies (Lowell *et al.* 1993) that brown adipose tissue deficiency might have profound effects in the physiology of these mice, specifically in their ability to thermoregulate body temperature and energy expenditure. However, most of the effects described by Lowell *et al.* (1993) in their mice were after 22-26 weeks of age. Before this period, transgenic and non-transgenic mice had similar food intake, and body weight although the latter had increased body lipids content. However, after 22-26 weeks of age, transgenic mice developed a marked hyperphagia with significantly high body weight, total body lipid content and increased lean body mass, which suggests that future experiments with CB1954 should also consider the analysis of these mice at a much older age.

## 7.3 CONCLUSION.

Experiments described in this section confirmed the ability of CB1954 to cross the transplacental barrier and more importantly confirmed the feasibility of *in utero* ablation of NTR expressing cells. Higher doses of CB1954 were found to cause toxicity to either the pups or the dam's ability to nourish the offspring. Nevertheless, lower doses of CB1954 decreased only the number of transgenic pups generated, which suggests a direct effect of the prodrug in those mice expressing the NTR. This lower dose of CB1954 produced two transgenic pups alive from a total expected of 14, as compared with the control untreated group. These two pups had a large reduction in the size of the brown adipose tissue, which was histologically active.

Decreased size and weight of pups born from dams that had been injected with the prodrug was also observed. It is believed that this effect corresponded to a premature delivery of the dams caused by stress produced during the injections since this effect was not observed in the control untreated dams.

CB1954 also caused a large decrease in the size of the testes in all mice whose dam had been treated with the prodrug, which might indicate a secondary longer-term effect of the prodrug in this organ. This effect may have important implications in the establishment of pedigrees from these mice since it is possible

that the reproductive capacity may have been affected, something that need to be addressed in future experiments.

No major physiological consequences were observed in any of the parameters measured with blood sugar levels and plasma triglycerides and FFA within normal ranges for all groups, which suggests that a partial ablation of brown adipose tissue has no major physiological consequence at least at this young age (8 weeks). This should not rule out the possibility of a longer-term effect, which remain to be determined.

These experiments encourage the belief that ablation of embryonic cells can be accomplished with this approach, which expand substantially the spectrum of opportunities of this system.

## 7.4 GENETIC ABLATION OF AN OBESE MOUSE: A POTENTIAL MODEL TO STUDY THE MORBID CONSEQUENCES OF DIABETES?

One of the diseases that can be best approached using transgenic models is obesity. Obesity refers to a heterogeneous group of disorders in which there is an excess of adipocyte cell mass and/or increased adipocyte cell number. This increase leads to a perturbation in the general metabolic state, and as a result, obesity is a major risk factor for a number of clinical diseases such as diabetes (NIDDM), cardiovascular disease, hyperlipidemia, and hypertension. In mice that inherit genes that cause them to become obese, triglycerides storage in adipose tissue is more efficient and catabolism of these stores is slower than in normal, non-obese counterparts. The phenotype of the obese mice is consistent with the hypothesis that metabolic abnormalities in diabetes are the result of an excess of adipose mass.

It was envisaged that incorporating the *ntr* transgene in the genetic background of an obese mouse could provide a novel approach to study the role of the WAT in the aetiology of diabetes. For this study it was decided to cross one of this transgenic lines expressing NTR in adipose tissue with the well characterised *ob/ob* model, since these mice do not have detectable levels of leptin and therefore any effect observed as result of the ablation of the adipose tissue could be attributed to the direct consequence of the reduction in fat mass. Thus, this could prove that the excess of fat is causing the metabolic abnormalities in these mice.

The obese mice (*ob/ob*) are characterised by a marked obesity, increased food intake (hyperphagia), a low body temperature and low physical activity thus, abnormalities in both energy intake and energy expenditure combine to produce the obese phenotype. Other characteristic of these mice includes hyperglycaemia, and markedly elevated plasma insulin levels associated with an increase in number and size of the islets of Langerhams (Coleman *et al.* 1978). Therefore, these mice represent a good model for the study of Type II diabetes mellitus, a principal cause of morbidity and mortality in human populations.

## 7.5 GENERATION OF $LEP^{OB}/LEP^{OB}$, Tg/+ MICE.

The mutation in the obese mouse is autosomal recessive and homozygous mutants are infertile. Therefore, mutant homozygous mice are obtained by mating known heterozygous mice. Breeding of these mice involved two generation crosses. In the first generation three males heterozygous for the obese gene, $Lep^{ob}/+$ (C57Bl/6J Harlan UK Limited) were crossed to transgenic females of the line 87 (tg/+) to produce double-heterozygous ($Lep^{ob}/+$, tg/+) mice. The same males ($Lep^{ob}/+$) were also crossed to F1 females (C57BLxCBA) mice to produce non-transgenic heterozygous ($Lep^{ob}/+$, +/+) offspring. In the second generation males and females from each group were intercrossed with the respective males and females of the other group. Intercrossed of these mice generated homozygous mutant for the obese genotype and heterozygous for the transgene ($Lep^{ob}/Lep^{ob}$, Tg/+) thus avoiding the generation of obese mice homozygous for the transgene, for which the effects of the ablation were unknown. The mice were first genotyped for the transgene locus using specific primers that detect a 492 bp band corresponding to the *ntr* transgene (as described in chapter 4) and then for the *Lep* locus. *Lep* genotyping involved two separates PCR reactions (primer sequences and PCR conditions are described in section 2.8.1 in chapter 2), one mutant-specific and one wild-type specific according to the method described by Anand and Chada, (2000). The mutant specific PCR generated a 295 bp band from the *Lep* allele, but no product from the wild-type allele, and vice versa for the wild type-specific PCR (Figure 7.6). The frequencies observed in the first and second generation crosses are shown in tables 7.4 and 7.5, respectively. Frequencies of transgenic mice in the first and second generations were at the expected Mendelian ratio, 47% in each case (Tables 7.4 and 7.5). However, the observed ratio for the heterozygous mutant genotype was much lower than expected. The percentage of ($Lep^{ob}/+$, tg/+) mice was 11.3% from an expected Mendelian ratio of 25% (Table 7.4). The same effect was observed in the second group from the cross ($Lep^{ob}/+$ x +/+) x (+/+, +/+) with a frequency observed for the ($Lep^{ob}/+$, +/+) mice of only 35% from a total expected of 50% (Table 7.4). It was later found that one of the $Lep^{ob}/+$ males used in the breeding had not transmitted the mutant gene to any of the offsprings in the first group of matings and to only 2 of the offsprings in the second group, therefore

explaining the differences in the expected frequencies that were observed. In the second generation however, observed frequencies were very close to the expected Mendelian ratio for each of the groups (Table 7.5), thus indicating no embryonic or neonatal lethality as result of these crosses.

Table 7.4 Observed frequencies for the crosses (*Lep*$^{ob}$/+, +/+ x +/+, tg/+) and (*Lep*$^{ob}$/+, +/+ x +/+, +/+).

| (*Lep*$^{ob}$/+, +/+) x (+/+, tg/+) | Expected Frequency | Observed Frequency | Total |
|---|---|---|---|
| *Lep*$^{ob}$/+, tg/+ | 25.0% | 11.3% | 7 |
| *Lep*$^{ob}$/+, +/+ | 25.0% | 21.0% | 13 |
| +/+, *tg*/+ | 25.0% | 35.5% | 22 |
| +/+, +/+ | 25.0% | 32.2% | 20 |
| Transgenic | 50% | 47% | **62** |
| (*Lep*$^{ob}$/+ x +/+) x (+/+, +/+) | Expected Frequency | Observed Frequency | Total |
| *Lep*$^{ob}$/+, +/+ | 50% | 35% | 26 |
| +/+, +/+ | 50% | 65% | 49 |
| | | | **75** |

Table 7.5 Observed frequencies of the cross (*Lep*$^{ob}$/+, tg/+) x (*Lep*$^{ob}$/+, +/+) mice.

| | Expected Frequency | Observed Frequency | Total |
|---|---|---|---|
| *Lep*$^{ob}$/*Lep*$^{ob}$, tg/+ | 12.5% | 13.5% | 13 |
| *Lep*$^{ob}$/*Lep*$^{ob}$, +/+ | 12.5% | 8.4% | 8 |
| *Lep*$^{ob}$/+, *tg*/+ | 25.0% | 28.1% | 27 |
| *Lep*$^{ob}$/+, +/+ | 25.0% | 30.2% | 29 |
| +/+, tg/+ | 12.5% | 9.4% | 9 |
| +/+, +/+ | 12.5% | 10.4% | 10 |
| Transgenic | 50% | 47% | **96** |

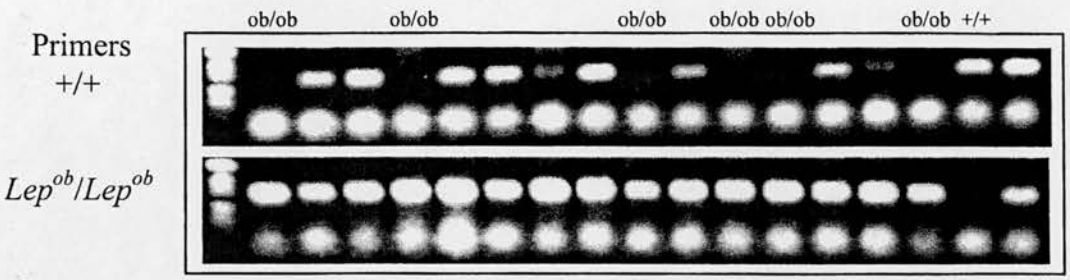
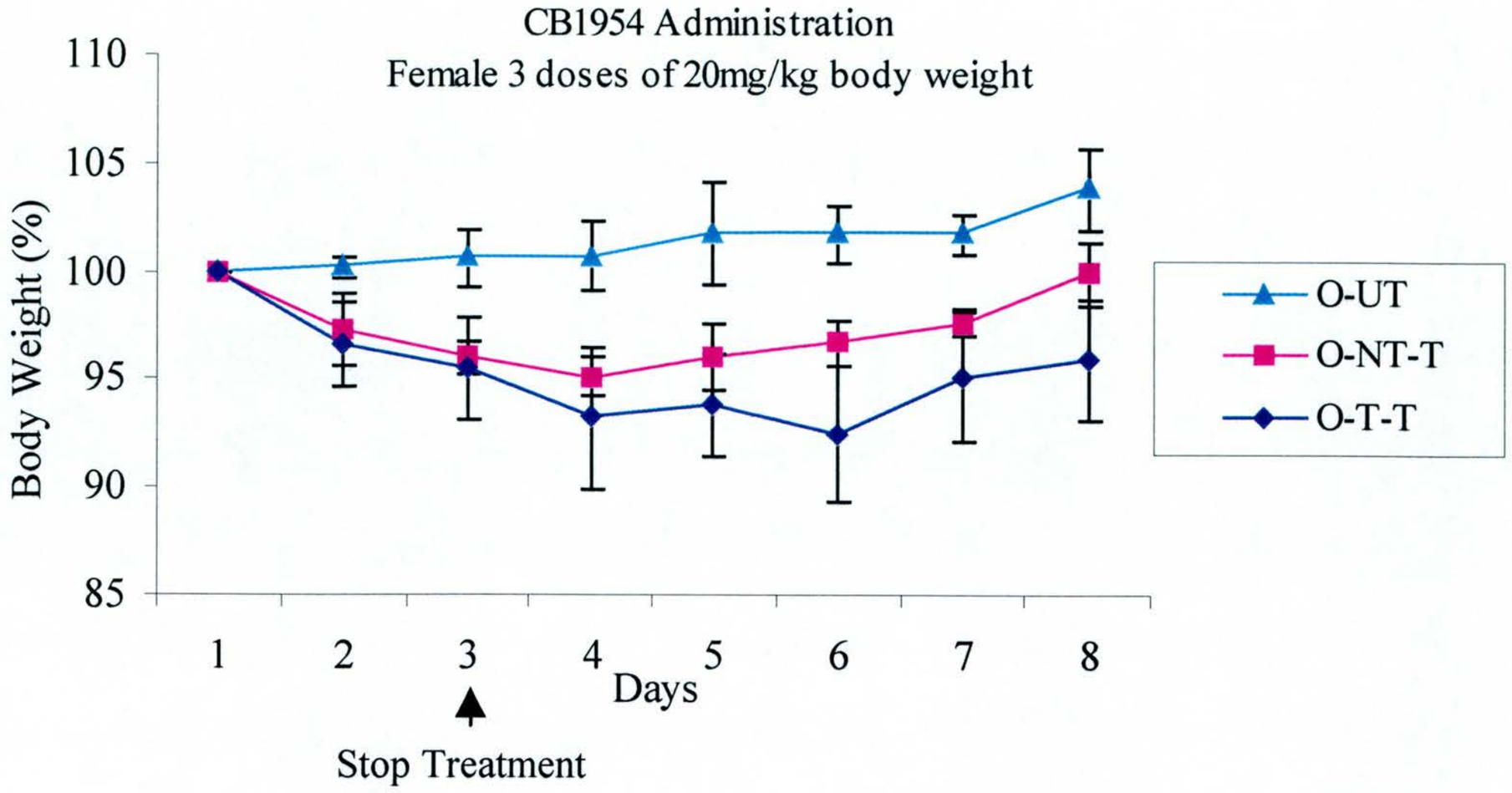



**Figure 7.6 Genotyping of obese mice from genomic tail DNA.**
1.5% agarose gel showing the results of a PCR reaction carried out with two set of primers to distinguish the obese genotype ($Lep^{ob}/Lep^{ob}$) from heterozygous ($Lep^{ob}/+$) or wild type (+/+).

Ablation experiments were carried out with mice housed at 23°C instead of 20°C as used in previous experiments since these mice have already a lower body temperature (Coleman *et al.* 1978). It was decided to use a dose of 20 mg/kg of body weight as mice injected previously with this dose showed no problems in withstanding the effects of the prodrug and the reduction of adipose tissue. With this regimen, transgenic male and female injected with the prodrug lost approximately 8% of body weight (Figures 7.7A and B, respectively) while control mice injected with the prodrug showed a reduction of only 4%. This data are quite similar to the reduction in body weight that was observed in previous experiments using similar dose-regimen in non-obese mice as described in chapter 5. Nevertheless, transgenic obese mice were more sensitive to the effects of the prodrug. These mice were observed hunched, cold and reluctant to move after the third day of the treatments with this regimen. A considerable reduction in the body temperature below the limit of detection of the instrument (<32°C) was observed in these mice, which is in contrast to the other control mice that had a body temperature of 35°C±0.5 (obese mice have already a lower body temperature). Despite the use of heated pads these mice did not recover and were sacrificed as soon as these signs were evident. Four out of 6 females and 2 out 3 males were sacrificed earlier (day 5 and 6) and one male was found dead on day 5. This mouse was not included in the final biochemical analysis.

A)

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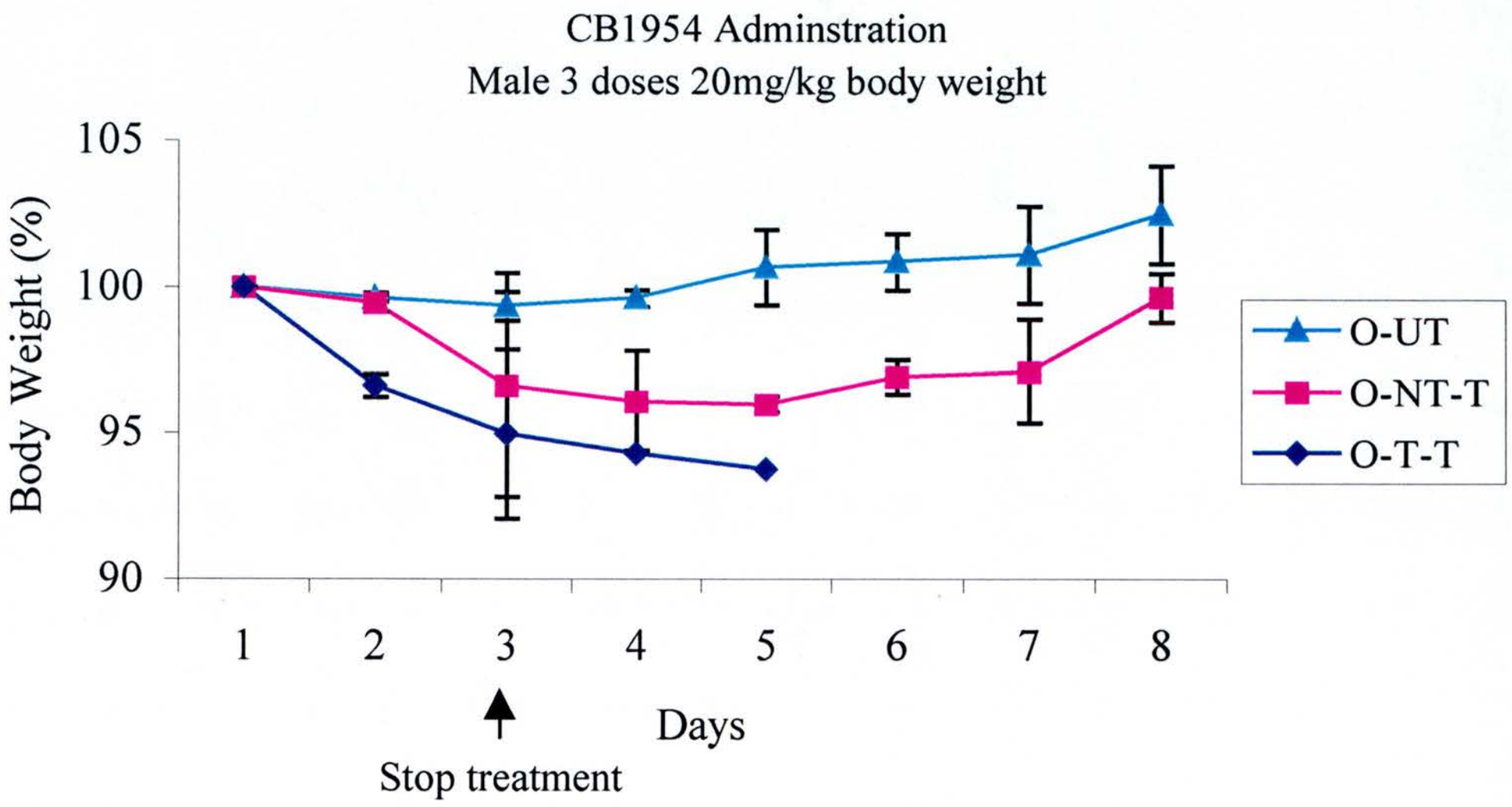


**Figure 7.7 Measurement of the body weight during treatment of obese mice with CB1954.**

10 weeks old obese-transgenic (O-T) and obese non-transgenic (O-NT) littermates were injected (T) with CB1954 at doses of 20 mg/kg of body weight for 3 days. Another obese non-transgenic group was left untreated (O-UT).

A and B females and males, respectively.

Dissection of these mice revealed some of the typical features of the obese mice. All major adipose deposits including subcutaneous, inguinal, retroperitoneal and gonadal were massively large. The livers were also large occupying most of the abdominal cavity and were pale indicating the massive accumulation of triglycerides. Analysis of transgenic mice injected with CB1954 also showed a large accumulation of adipose tissue in all different deposits. Nevertheless, analysis of the wet weight of these deposits showed a reduction of 20% in the gonadal and retroperitoneal fad pads of transgenic male compared with the control groups (Table 7.6). Transgenic females injected with the prodrug also showed a reduction of approximately 15% in the inguinal fat pad but this effect was not observed in the retroperitoneal fat pad (Table 7.7), which might be due to the fact that this deposit was dissected as one large deposit with the parametrial fat due to the complexity to separate both cleanly. Despite this reduction in fat mass, no significance difference could be observed when these deposits were analysed with the other groups, in part due to the large variation that was observed between individuals in each group. This large variation was also reflected in the size of each mouse, which despite being all littermates born during the same week, some of them were markedly heavier than others. This effect is more notorious for instance in the transgenic male group treated with the prodrug, which despite the loss of ~8% of body weight, these mice were still much heavier than the other two groups (Table 7.6). This effect might correspond to the different response of these mice to the developing disorders associated with their obesity or it might be due to some variation due to the genetic background (Coleman and Hummel, 1973).

The brown fat, as represented by the interscapular fat mass (BAT), was slightly decreased in both transgenic males and females, although this effect was not significant.

Table 7.6 Body composition of male obese mice.

| | Body Weight Grams | Gonadal (WAT) (%) | Retro (WAT) (%) | Intersc. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| O-T-T (n=3) | 48.9±3.1 | 3.8±0.05 | 1.7±0.09 | 0.33±0.01 | 6.7±0.4 | 0.61±0.01 | 0.11±0.02 | 0.40±0.06 |
| O-NT-T (n=2) | 47.1±2.1 | 4.6±0.87 | 2.1±0.27 | 0.42±0.03 | 5.9±1.2 | 0.52±0.01 | 0.14±0.02 | 0.35±0.02 |
| O-UT (n=2) | 45.0±0.8 | 4.4±0.20 | 2.2±0.35 | 0.36±0.02 | 7.6±0.4 | 0.63±0.13 | 0.17±0.01 | 0.38±0.01 |

Table 7.7 Body composition of female obese mice.

| | Body Weight Grams | Gonadal (WAT) (%) | Retro (WAT) (%) | Intersc. (BAT) (%) | Liver (%) | Kidney (%) | Spleen (%) | Heart (%) |
|---|---|---|---|---|---|---|---|---|
| O-T-T (n=6) | 45.2±0.8 * | 3.6±0.27 | 2.4±0.14 | 0.27±0.04 | 7.6±0.4 | 0.55±0.03 | 0.11±0.01 | 0.35±0.01 |
| O-NT-T (n=4) | 45.4±1.5 * | 4.2±0.22 | 2.5±0.08 | 0.32±0.04 | 5.8±0.4 | 0.52±0.02 | 0.18±0.01 | 0.35±0.02 |
| O-UT (n=2) | 51.2±1.7 | 4.2±0.31 | 2.6±0.53 | 0.27±0.04 | 7.8±1.1 | 0.45±0.01 | 0.14±0.06 | 0.29±0.04 |

P Value *=0.05

Data are mean ± S.E.M. Animals were obese transgenic and non-transgenic littermates of 14 weeks old. The organ weights were measured as a percentage of the animal's body weight. Probabilities (P) are from a ANOVA one way.
O-T-T: Obese transgenic treated.
O-NT-T: Obese non-transgenic treated.
O-UT: Obese untreated.

Histological analysis of this tissue in control mice showed sparse eosinophilic staining, a single lipid droplet per cell, and peripheral nuclei, resembling the appearance of white adipose tissue (Figure 7.8 A). This is the typical appearance of inactive brown adipose tissue (BAT) as seen in most of the obese models (Trayhurn P, 1986), which indicates that BAT of these mice is atrophied and thermogenically quiescent. This atrophy includes selective losses of uncoupling protein (UCP) and lipoprotein lipase, which explain the lower body temperature that is observed in these mice (Himms-Hagen, 1990). This is in marked contrast to the appearance of active BAT as seen in wild type mice (Figure 7.5 as an example in this chapter). The appearance of the brown fat in transgenic mice treated with the prodrug was similar to that of control mice, nevertheless in these sections there was a marked disappearance of adipose cells leaving some empty spaces that were occupied by stroma (Figure 7.8 B).

Histological analysis of the white adipose tissue showed no major difference between the groups (not shown) possibly due to the less defined structure of this deposit and/or due to the higher capacity of hyperplasia and hypertrophy of these cells in the obesity condition. Nevertheless, histological analysis of the skin confirmed a considerable reduction in the adipose cells of some of the transgenic mice that were treated with the prodrug (Figures 7.8 C and D). This effect was again more notable in females than in males thus confirming the previous observation (chapter 5) that made reference to the fact that females have more fat in the skin than males.

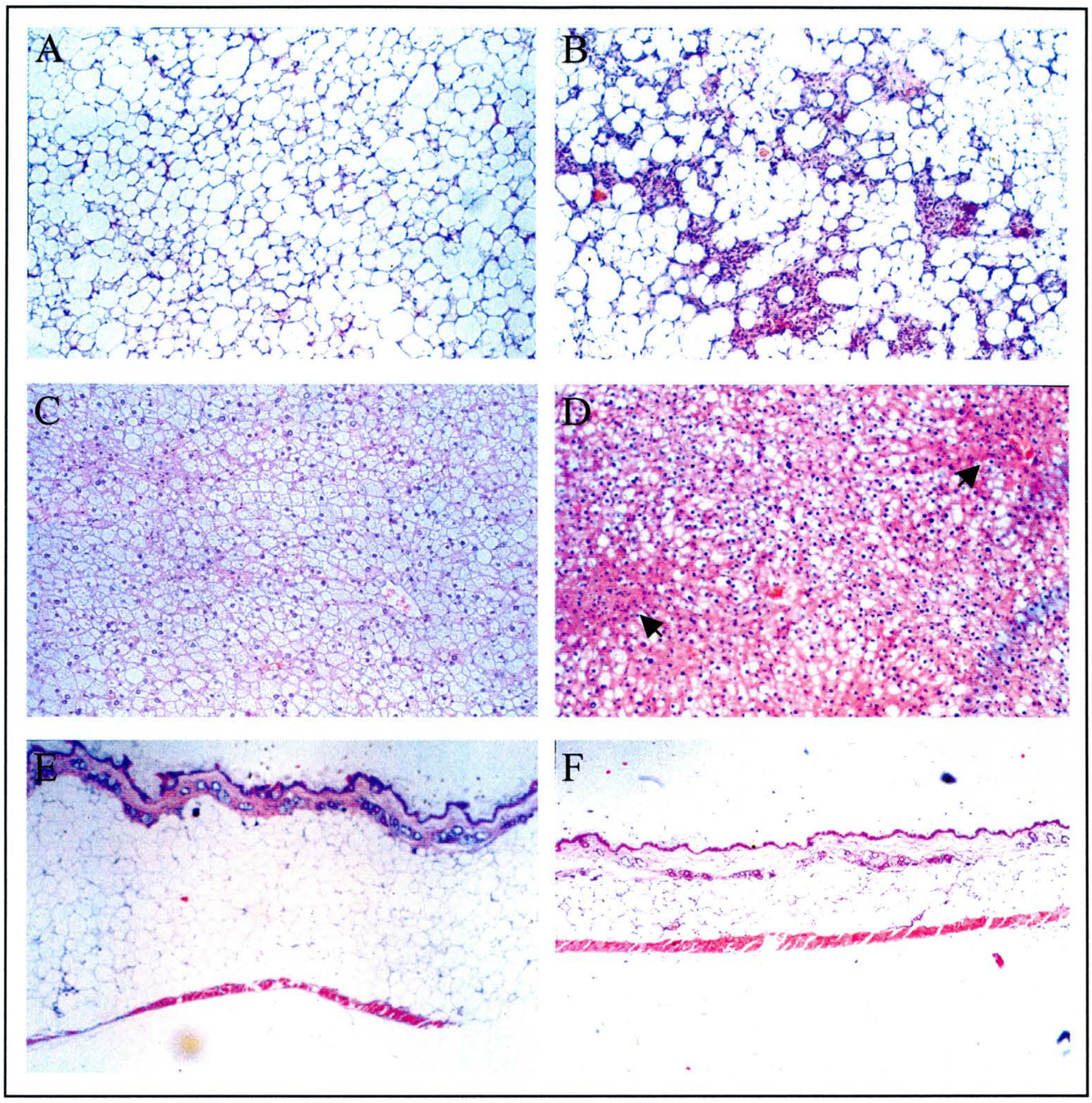


**Figure 7.8 Histology of different tissues in obese mice after treatment with CB1954.**

Histological sections of the interscapular brown fat (A and B), liver (C and D) and skin (E and F) from 7 weeks old female transgenic (B, D, F and H) and non-transgenic (A, C, E and G) littermates.

Tissues were fixed in NBF and paraffin-embedded sections stained with haematoxylin and eosin. Magnification: 100X A,B,C, and D; 40X E and F.

Comparison of the other organ weights showed no major difference with the other groups, although a decrease in the steatosis of some of the transgenic mice that had been treated with the prodrug was observed (Figure 7.9). Histological analysis of sections of these livers confirmed this observation. The liver of obese control mice, showed hepatocytes that were swollen, stained palely eosinophilic and contained numerous vacuoles, which coalesced to form distinct unilocular vacuoles displacing the nucleus to the cell periphery (Figure 7.8 C). The livers of some obese transgenic mice injected with the prodrug, showed a marked reduction of vacuoles, which stained more eosinophilic and there were some areas with better organised hepatic cords (see arrows in Figure 7.8 D). It has been described that excessive caloric intake might play a role in the genesis of fatty liver disease observed in obese mice (Sheth *et al.* 1997). Therefore it is important to determine if CB1954 beneficial effect on fatty liver disease is the direct result of a decrease in adipose tissue or the indirect effect of the prodrug in reducing the excessive caloric intake (hyperphagia) normally observed in these mice. Food intake analysis of these mice while treated with the prodrug should clarify this point in future experiments.

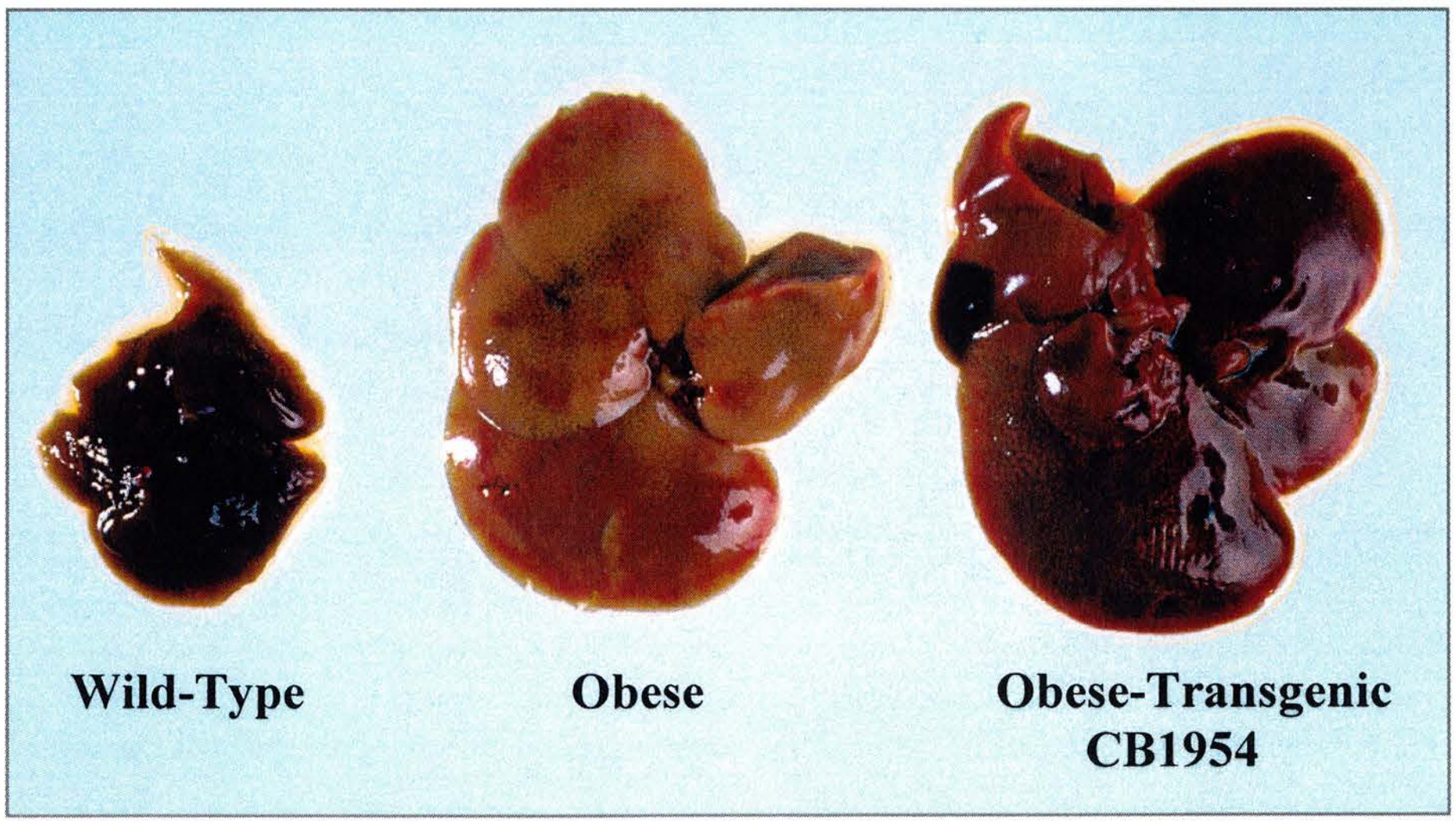


**Figure 7.9 Photomicrograph of livers.**
Photomicrograph of livers that show the marked decrease in fatty acids accumulation in one of the transgenic mice that was injected with the prodrug (right side), compared to the control obese mouse (middle in picture). Although both livers of the obese mice show hepatomegaly as compared with the wild-type control on the left side.

One of the main objectives with these experiments was to assess the consequences of the ablation of adipose tissue in the physiological parameters already altered in obese mice. Unfortunately, this could not be established due to a problem that arose three days before remaining transgenic and control mice were going to be sacrificed (day 8). The temperature of the room, where the mice were normally located dropped to 14°C due to a failure in the system. This problem was not detected until all mice were sacrificed and blood glucose analysis carried out, otherwise the termination of the experiments could have been postponed for a few days later thus allowing these mice to settle down again. The first indication that something was seriously wrong came from the results of the blood glucose analysis carried out in these mice that plummeted in all mice to levels similar to those observed in wild type mice (90 mg/dl) although there were a few mice which still had relatively high glucose levels (>200 mg/dl). This is in marked contrast to the non-fasting values expected for obese mice that are around 500mg/dl. In fact, blood glucose levels in those transgenic mice that were sacrificed earlier (day 5 and day 6) showed values in this order of magnitude (~500 mg/dl) although some of them showed a reduction in blood glucose levels (280-300mg/dl). Nevertheless, these values are still much higher than those observed at day 8 when all the control mice were sacrificed.

This failure in the heat system of the room also had implications with other parameters such as insulin. Insulin was also decreased in these mice although there was more variation in this parameter with some mice having low levels (~12ng/ml) and others high (>70ng/ml). This is in marked contrast to what is normally observed in obese mice, which are characterised by high insulin and glucose levels.

The drop in these physiological parameters that was observed in these mice suggests that due to the low temperature of the room these mice may have been starving or unable to feed properly. Both glucose and insulin levels are consistent with this observation. A drop in insulin drives the metabolic adaptation to fasting and adipose tissue triglycerides are hydrolysed which releases free fatty acids that are metabolised by the liver to produce ketone bodies constituting the main fuel for the brain. Although it would have been interesting to measure the levels of ketones, unfortunately no more blood was available to carry out this assay. Nevertheless,

analysis of free fatty acids in pooled samples of plasma confirmed a slight increase in these levels (Table 7.8).

Table 7.8 Physiological characteristic of obese mice.

| | Glucose mg/dl | Triglycerides mg/dl | Insulin ng/ml | FFA mM |
|---|---|---|---|---|
| O-T-T | 383±217 (88-612) | 285±330 (69-929) | 58.3±49.5 (4.7-120) | 0.58±0.02 |
| O-NT-T | 153±48 (110-235) | 57±14 (41-67) | 40±41 (10-69) | 0.81±0.21 |
| O-UT | 168±76 (83-234) | 72±18 (58-98) | 27±19 (12-55) | 1.12±0.07 |

Data are means ± SD. Animals were 14 weeks old males and females. Glucose, triglycerides and insulin analyses were carried out with plasma from each animal. Free fatty acid analysis was carried out with pooled samples from 3-5 mice.
O-T-T: Obese transgenic treated.
O-NT-T: Obese non-transgenic treated.
O-UT: Obese untreated.

Although a reduction in both glucose and insulin levels could be observed in some transgenic mice treated with the prodrug that had been sacrificed earlier, it is impossible to confirm whether this effect was the direct result of the decrease in adipose tissue, since no possible comparison can be made with any of the control mice. If ablation of adipose tissue really improved glucose homeostasis in these mice will have to be confirmed with further experiments.

Another effect observed only in those transgenic mice treated with the prodrug was a large increase in the levels of triglycerides of up to 10 fold (900mg/dl), although this effect was not observed in all mice, since others showed levels similar to those observed in the control groups (80mg/dl). This high level of plasma triglycerides could indicate a destination for the fat in the ablated adipocytes. Although previous experiments, as described in chapter 6, showed only a slight increase in the levels of plasma triglycerides in non-obese transgenic mice, the difference might be in the higher content of fat in the adipocytes of obese mice.

Another possibility for this difference is that fatty acid are oxidised more efficiently in or near adipocytes in wild type mice thus preventing them from reaching the liver, the site of ketogenesis. If so, one might expect increased expression of enzymes of fatty acid oxidation such as CPT-1 and ACO in adipose tissue during the disappearance of fat (Zhou *et al.* 1999). Future experiments measuring the expression of these enzymes should clarify this observation.

## 7.6 CONCLUSION.

To understand better the contribution of the adipose tissue to diabetes and metabolism, it would be valuable to examine a mouse in which the adipose tissue has been removed at defined time points in the life of the animal. Such a mouse model would allow a functional dissection of obesity and its metabolic sequel to be studied. These preliminary experiments showed the feasibility to genetically ablate adipose cells in obese mice expressing the *E.coli ntr* gene in the adipose tissue. Histological analysis of paraffin-embedded tissues stained with haematoxylin and eosin provided direct evidence of the change in tissue morphology, with a more marked effect being observed in the adipocytes of the skin. Although the magnitude of cell ablation was not as marked as in previous experiments involving wild-type mice, this may reflect the higher capacity of hypertrophy and hyperplasia of adipocytes observed in the obesity condition.

A decrease in the levels of glucose and insulin was observed in transgenic mice treated with the prodrug, which could suggest an improvement in the glucose homeostasis and hence in the diabetes of these mice. Nevertheless, a similar effect observed in control mice, due presumably to starvation caused by a decreased in the temperature of the room spoils any comparison with these mice and preclude any reliable conclusion. That the effect in transgenic mice was the direct result of the reduction of adipose tissue mass will remain to be determined.

A decreased in the steatosis was also observed in some transgenic mice after treatment with the prodrug. That this effect corresponds to a direct result of the decrease in the adipose tissue or an indirect effect of the prodrug, reducing the food intake of these animals, also remains to be determined. As well is yet to be

demonstrated whether reducing the fat content of obese mice would lead to a reestablishment of the metabolic parameters and disappearance of some of the morbidity consequences of obesity. Nevertheless, these preliminary results presented in this chapter should encourage further studies to find the answers to these questions.

# 8. ALTERNATIVE SYSTEM OF CELL ABLATION MEDIATED BY RAIDD.

## 8.1 BACKGROUND.

Control of cell number depends primarily on the balance between processes of proliferation and apoptosis. In mammals, apoptosis begins at the blastula stage and continues throughout life and can be as equal important in controlling cell numbers, as is cell proliferation. The RAIDD/CRADD gene, a protein that serves as an adapter for death proteases in the apoptotic signalling pathway, was recently isolated (Horvat and Medrano, 1998) as a potential candidate for the *hg* (high growth) gene in the mouse. The *hg* gene was identified in a selection experiment for rapid growth. It produces a 30-50% increase in weight gain of homozygous individuals without resulting in obesity. The human death adapter molecule RAIDD/CRADD, which share very high homology with the mouse, was recently tested in MCF-7 cell lines (a human breast carcinoma cell) to address the function of RAIDD in apoptosis. MCF-7 cells transiently transfected with a RAIDD construct underwent apoptosis, which was inhibited by the inclusion of several known inhibitors of apoptosis, thus confirming the role of this gene in the apoptotic pathway (Duan and Dixit, 1997). RAIDD (RIP-associated ICH-1 homologous protein with a death domain) is an adapter molecule that contains an amino-terminal CARD (Caspase Recruitment Domain) region and a carboxy-terminal "death domain". It mediates the action of cystein proteases involved in the apoptosis pathway (Duan and Dixit 1997). The carboxy-terminal domain combines with the homologous death domain region in RIP, a serine/threonine kinase component of the TNFRI signaling complex, and the Caspase-2 (ICH-1) molecule associates with the homologous amino-region of RAIDD (Cohen G, 1997).

This chapter describes preliminary experiments carried out with a preadipocyte cell line (3T3L1) overexpressing the murine RAIDD cDNA in an attempt to test the feasibility of the RAIDD-mediated ablation in these cells. A range

of phenotypes was observed after a number of individual clones transfected with the RAIDD cDNA were exposed to the cocktail of hormones that induce the conversion of these cells into adipocytes.

The most consistent effect was a complete blockage of the differentiation of these cells into adipocytes, which was observed with a number of independently isolated clones. This effect was judged by the absence of lipid accumulation, a lack of expression of adipocytes-specific genes and a fibroblastic morphological appearance. Others clones showed impaired differentiation, as judged by the partial accumulation of lipid and the death of differentiated adipocytes that escaped the initial blockage. A possible mechanism for this RAIDD-mediated effect is discussed.

## 8.2 TRANSGENE CONSTRUCTS.

### 8.2.1 GENERATION OF THE aP2-pA CONSTRUCT.

Figure 8.1 corresponds to a diagram that summarises the two cloning steps used to generate the aP2-driven RAIDD plasmid. In the first step, a 0.85 kb fragment containing the pA signal from SV40 was excised from the pA plasmid (kindly provided by Dr. Wei Cui) by double digestion with *Sma* I/*Not* I and gel purified ligated to the aP2 Promoter plasmid also digested with *Sma* I/*Not* I. For the confirmation of the appropriate clone, minipreps were made of DNA from several colonies and the aP2-pA fragment liberated from the backbone vector by double digestion *Sal* I/*Not* I. In this way, two bands were expected for those clones that had incorporated the pA fragment, a 6.25 kb band containing the aP2 plus pA fragments and a 2.9 kb band corresponding to the backbone vector (Figure 8.2 B).

### 8.2.2 GENERATION OF THE aP2-RAIDD-pA CONSTRUCT.

The second step of this cloning (Figure 8.1 B) consisted in the incorporation of a 0.88 kb fragment of the RAIDD cDNA. This fragment was excised from the plasmid pB308 (kindly provided by Dr. Simon Horvat) by double digestion with

*Sma* I/*Cla* I (Figure 8.3 A) replacing the pA signal for that of SV40, which has been observed to work well in our lab and end filled ligated into the unique *Sma* I (CIP treated) site of the newly generated aP2-pA plasmid. Confirmation of the appropriate integration of this DNA came from minipreps of DNA from colonies digested with *Sal* I/*Not* I. In this way, two bands were expected for those clones that had incorporated the RAIDD fragment, a 7.1 kb band corresponding to the aP2-RAIDD-pA fragment and a 2.9 kb band of the backbone vector (Figure 8.3 B). Further analysis was necessary to confirm for the right orientation of the RAIDD fragment, which had been incorporated as a blunt end. Confirmation of the right orientation came from a series of restriction digest of 5 clones that were chosen at random from Figure 8.3 B. Digestion of these clones with *Sma* I/*EcoR* I would have generated three fragments of 1.7 kb, 3.7 kb and 4.69 kb if RAIDD was in the right orientation (see lanes 12 and 14 in Figure 8.4) or three fragments of 2.58 kb, 3.7 kb and 3.81 kb if it was in the wrong orientation. *Apa* I also served to look for the appropriate orientation although due to the poor quality of the DNA (minipreps DNA), it was not as good as with *Sma* I/ *Eco R* I.

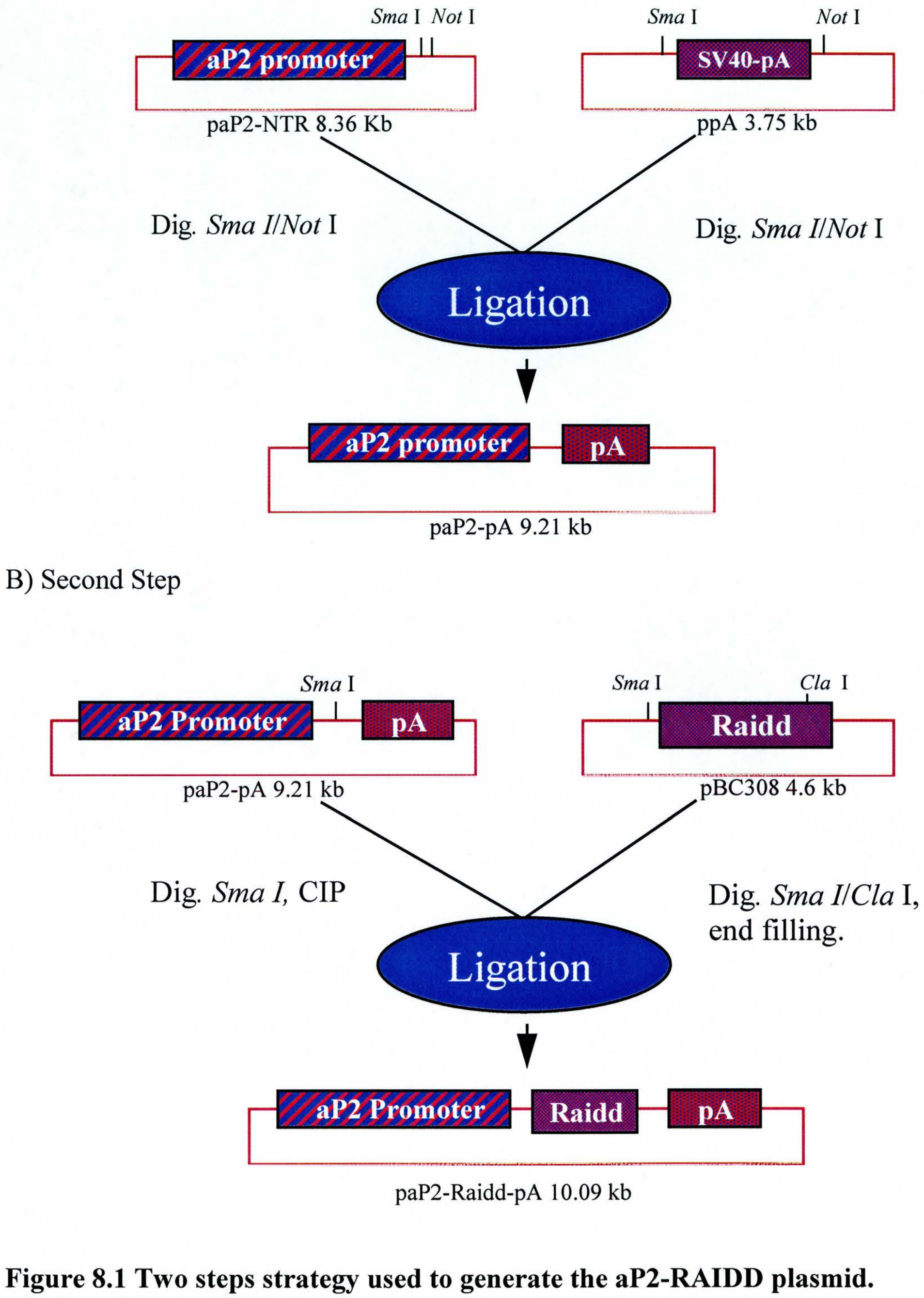


**Figure 8.1 Two steps strategy used to generate the aP2-RAIDD plasmid.**
A) First Step: 0.85 kb fragment containing the pA signal was excised from the plasmid pSV40-pA by double digestion with *Sma* I/*Not* I and ligated into the aP2 promoter plasmid also digested *Sma* I/*Not* I.
B) Second Step: 0.88 kb fragment of the RAIDD cDNA was excised from the plasmid pB308 by double digestion with *Sma* I/*Cla* I and end filled ligated into the aP2-pA plasmid also digested *Sma* I.

1) First Step: Cloning aP2-pA.

A) B)

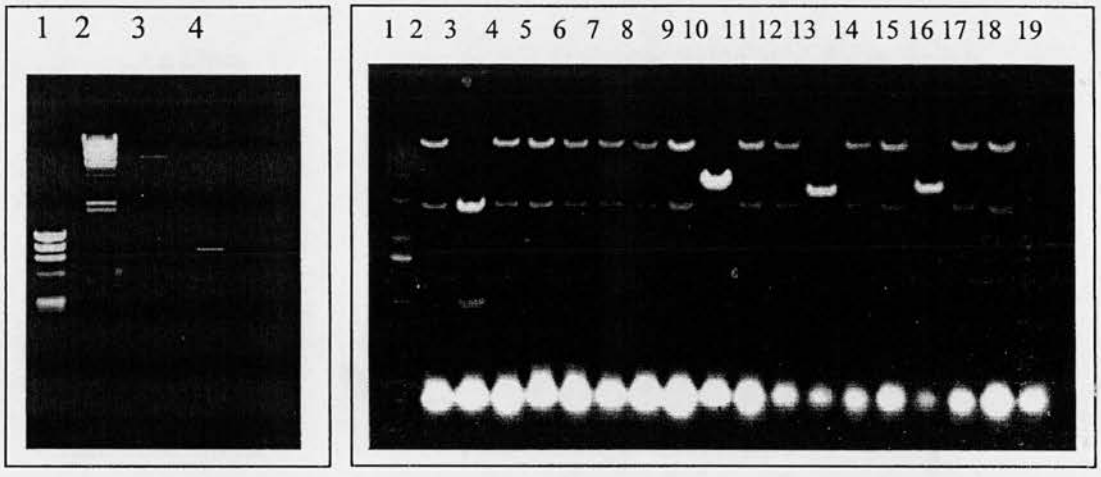


**Figure 8.2 Restriction digest of plasmids used to generate the aP2-pA plasmid.**
A) Lanes 1 and 2 correspond to ϕ–X 174 *Hae* III and λ *Hind* III markers, respectively. Lane 3 corresponds to the 8.36 kb fragment of the linearised aP2 promoter plasmid digested *Sma* I/*Not* I. Lane 4 corresponds to the 0.85 kb fragment of the SV40 plasmid digested *Sma* I/*Not* I.

B) Lane 1 corresponds to the 1 kb marker. Lanes 2-18 correspond to DNA from different putative colonies digested *Sal* I/*Not* I. A number of clones seem to have incorporated the pA fragment as evidenced for the 2 different bands of the expected size (6.25 kb aP2-pA+2.9 kb plasmid band).

2) Second Step: Cloning aP2-RAIDD-pA.

A) B)


**Figure 8.3 Restriction digest of plasmids used to generate the aP2-RAIDD-pA.**
A) Lane 1 corresponds to λ *Hind* III marker. Lane 2 correspond to the 9.2 kb fragment of the linearised aP2-pA plasmid (generated above) digested *Sma* I. Lane 3 corresponds to the 0.88 kb fragment of the B308 plasmid digested *Sma* I/*Cla* I.
B) Lanes 1 and 20 correspond to 1 kb marker. Lanes 2-19 correspond to DNA from different putative colonies digested *Sal* I/*Not* I. A number of clones seem to have incorporated the insert (RAIDD fragment) as evidenced for the size of the two expected bands (7.1 kb aP2-RAIDD-pA+2.9 kb plasmid bands). Confirmation of the orientation of the RAIDD fragment (blunt end ligated) came from the digests in Figure 8.4.


**Figure 8.4 Restriction digest of 5 putative clones.**
Lanes 1 and 18 1 kb marker. Lanes 2 and 17 λ *Hind* III marker. Lane 3 aP2 Promoter plasmid *Sal* I/*Not* I. Lane 4 aP2-pA plasmid *Sma* I/*Not* I. Lane 5 aP2-pA plasmid *Apa* I. Lanes 6, 7, 8, 9 and 10 correspond to putative clones 2, 7, 9, 10 and 12 digested *Apa* I, respectively. Lane 11 aP2-pA *Sma* I/*Eco* R I, lanes 12, 13,14, 15 and 16 correspond to putative clones 2, 7, 9, 10 and 12 digested *Sma* I/*Eco* R I, respectively.


**Figure 8.5 Restriction map of the paP2-RAIDD plasmid.**
A=*Apa* I, Sa=*Sal* I, E=*EcoR* I, S=*Sma* I, N=*Not* I.

| | |
|---|---|
| *Sma* I digest: | 10.09 kb |
| *Sal* I digest: | 10.09 kb |
| *Not* I digest: | 10.09 kb |
| *Sma* I/*EcoR* I | 1.7 kb + 3.7 kb + 4.69 kb |
| *Apa I* digest: | 4.04 kb + 6.04 kb |

aP2 Promoter: 5.4 kb, RAIDD: 0.88 kb pA: 0.85 kb and backbone plasmid 2.96 kb (pSK II).
Also in Figure 8.4 lanes 3, 4, 5 and 11 correspond to control digestions. Thus, in lane 3 *Sal* I/*Not* I digest 5.4 kb and 2.96 kb bands in aP2 Promoter plasmid. Lane 4 *Sma* I/*Not* I digest 0.85 kb and 8.36 kb bands in aP2-pA plasmid. Lane 5 *Apa* I digests 9.21 kb in aP2-pA plasmid and lane11 *Sma* I/*Eco* R I digest 1.7 kb, 3.7 kb and 3.81 kb bands in aP2-pA plasmid.

## 8.3 OVEREXPRESSION OF MURINE RAIDD cDNA BLOCKS DIFFERENTIATION OF TRANSFECTED 3T3L1 CELLS INTO ADIPOCYTES.

Microscopic examination showed that when 3T3L1 cells are induced to differentiate in the presence of agents that promotes differentiation (dexamethasone, insulin, methylisobutylxanthine) these cells undergo a dramatic morphological change that ends with the massive accumulation of cytosolic triglycerides (Figure 3.1 in chapter 3 and Figure 8.6 E below). In marked contrast, different clones of 3T3L1 cells stably transfected with a plasmid containing the murine RAIDD cDNA, failed to accumulate cytosolic triglycerides when treated under identical conditions as the parental cells. The effect on these cells was evidenced by light microscopy (Figure 8.6) and oil Red O staining, which stains specifically the lipid droplets (Figure 8.7). Similar results were obtained in a second transfection experiment with 4 other independent clones. This was true even when these cells were left for over 2 weeks after induction to differentiate or when induced to differentiate after 4 or 6 days postconfluence instead of 2 days recommended in the differentiation protocol (Student *et al.* 1980).

Two clones of 3T3L1 cells transfected only with the backbone plasmid containing the antibiotic resistance marker neomycin, differentiated normally (data not shown). Furthermore, the same backbone plasmid had been used before in the experiments described in chapter 3 where more than 12 independent clones transfected with the NTR gene were expanded and differentiated showing no problem in their ability to convert into adipocytes, thus arguing against an indirect effect of the plasmid sequences in this blockage.

Other effects observed with some of the clones were a partial differentiation into adipocytes as judged by the reduction in the accumulation of cytoplasmic triglycerides (Figure 8.6 A), although these cells underwent a morphological change from fibroblast-like to rounded up cells. Another clone showed a similar reduction in the accumulation of triglycerides but partially differentiated adipocytes that have escaped the initial blockage begun to die by a mechanism which, although was not studied, it is believed to have corresponded to apoptosis due to the proposed role of this gene in the apoptotic pathway (Duan and Dixit, 1997).

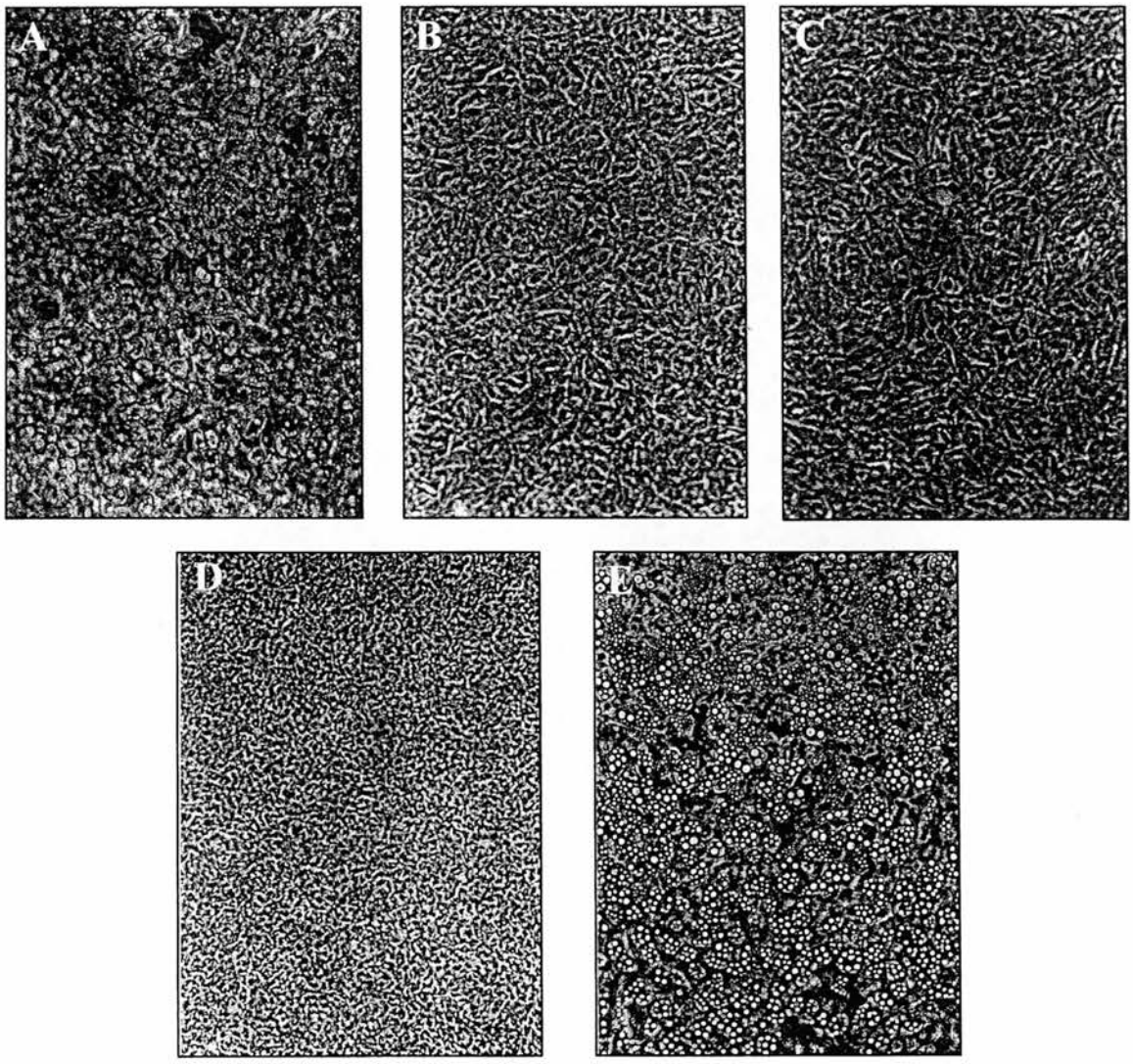


**Figure 8.6 Effects of the overexpression of RAIDD on the differentiation of 3T3L1 cells under optimal hormonal conditions.**
Phase-contrast photomicrograph of different clones of 3T3L1 cells transfected with RAIDD. Cells were subjected to differentiation with standard differentiation medium as described earlier on chapter 3. Panel A shows a clone that differentiated into adipocytes but has accumulated less triglycerides. Panels B, C, and D show some of the clones that were completely blocked and thus, did not differentiate. Panel E corresponds to the control parental cells. Magnification: 100X A, B, C and E; 40X D.

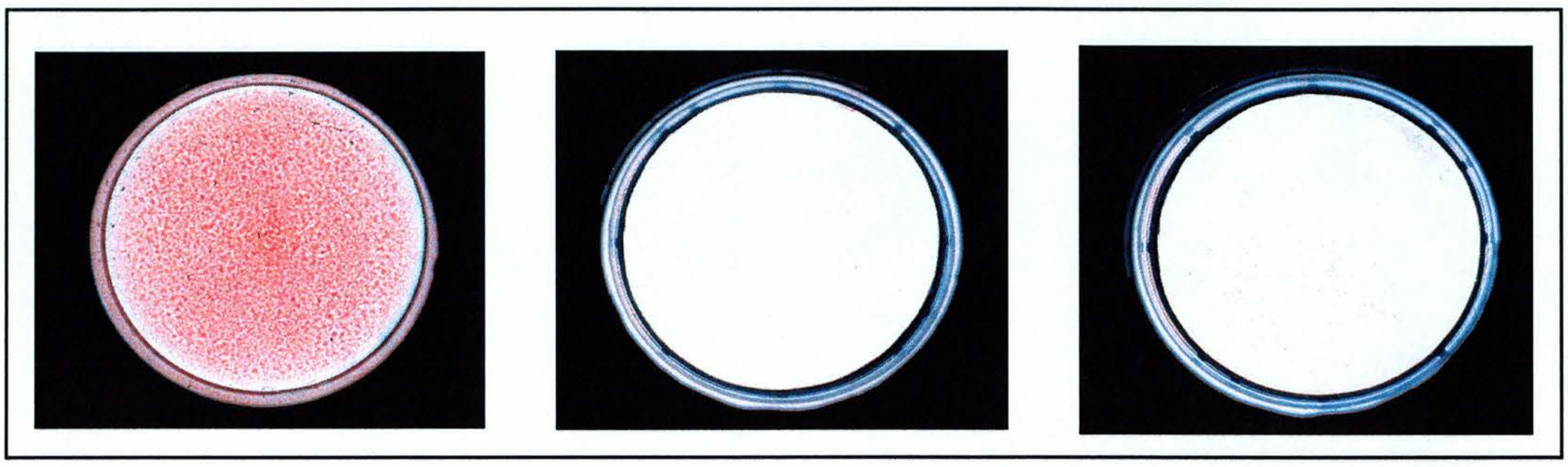


**Figure 8.7 Lipid Staining.**
At 8 days following differentiation, cultures of two different clones (clones 2 and 6) transfected with RAIDD and untransfected parental cells (control) were fixed with 10% paraformaldehyde in PBS, stained for lipid content by Oil Red O, and photographed.
Red dish corresponds to cells that show substantive accumulation of triglycerides indicating appropriate differentiation into adipocytes. No staining is observed in the two clones shown in this figure.

## 8.4 PRELIMINARY ANALYSIS BY RT-PCR OF THE EXPRESSION OF mRNAs FOR TARGET GENES IN THE DIFFERENTIATION OF ADIPOCYTES.

Expression of exogenous and endogenous RAIDD and a number of genes involved in the differentiation of 3T3L1 into adipocytes were assessed by RT-PCR from total RNA made of 6 of these clones transfected with RAIDD after 8 days of the induction to differentiate with the cocktail of hormones. Four of these clones did

not differentiate into adipocytes (clones 2, 3, 5 and 6 in Figure 8.8). Two other clones differentiated into adipocytes but they accumulated less cytosolic triglycerides (clone 1 see Figure 8.6 A above) and/or once differentiated, adipocytes begun to die (not shown). As control, total RNA from parental cells before differentiation (P) and after 8 days of differentiation (A) was also included. Five μg of total RNA (DNase-treated) was reverse transcribed as described in chapter 2, and expression of these genes assessed by amplification of the respective cDNA using two specific primers that were selected from the published sequence (Pub Med) using the public domain software Primer Design.

In order to distinguish expression of exogenous from endogenous RAIDD, a pair of primers was designed to amplify a region containing the transcribed +21 bp sequence of the aP2 promoter (5' primer) and a reverse primer corresponding to an internal sequence of RAIDD (3' primer). Amplification of endogenous RAIDD with an internal 5' primer and the reverse primer as above detects both endogenous and exogenous RAIDD (primer sequences and PCR conditions are described in chapter 2). Thus, expression of exogenous RAIDD would yield a band of approximately 620 bp with the first set of primers and expression of both endogenous and exogenous RAIDD would yield a band of approximately 502 bp with the second set of primers.

Figure 8.8 A confirmed expression of the exogenous RAIDD in all clones transfected with the RAIDD cDNA. As expected, control parental cells before differentiation (P) and after differentiation into adipocytes (A) showed no signal for the exogenous RAIDD. Analysis with the set of primers that detects the endogenous and exogenous RAIDD shows lower expression of some clones, which might simply indicate lower yield of the transcription from total RNA to cDNA (Figure 8.8 B). This observation is further confirmed from the semi-quantitative PCR analysis carried out with GAPDH as an internal control and endogenous/exogenous set of primers (Figure 8.8 C).

The inability of 3T3L1 cells transfected with the murine RAIDD cDNA to express the differentiated phenotype was further substantiated by analysing the expression of the differentiation-specific marker adipsin. Adipsin is a serine protease

whose cDNA was isolated by differential screening of preconfluent and differentiated 3T3L1 cells (Cook *et al.* 1987). It is found *in vivo* in the circulation of both animals and humans and is produced and secreted *in vitro* by differentiated 3T3L1 cells thus, making this gene a marker for terminally differentiated adipocytes. RT-PCR analysis carried out from total mRNA transcribed from these clones showed a strong signal only in those clones that differentiated into adipocytes (clones 1 and 4) and in the control parental cells (3T3L1) after differentiation into adipocytes (A) (Figure 8.8 D). In contrast, no induction of the expression of this gene was observed for the clones 2, 3, 5 and 6 thus confirming the block in the differentiation of these clones.

While a number of mRNAs, whose levels increase during adipocytes differentiation have been well characterised and their regulation has been studied in detail, very few have been identified that are down-regulated during adipocyte differentiation. Preadipocyte factor 1 (Pref-1) corresponds to one of such genes whose mRNA levels decrease during adipocyte differentiation. Pref-1 was discovered after differential hybridisation screening of preadipocytes 3T3L1 and corresponds to a cell surface protein that appears to have a role in maintaining preadipocytes in the undifferentiated state (Smas and Sul 1993).

Constitutive expression was shown to inhibit adipogenesis, confirming a regulatory role in adipocyte differentiation (Smas *et al.* 1994). As Pref-1 down regulation is required for adipocyte differentiation, this should be reflected by increased expression of mRNA in cells or culture conditions in which adipocyte differentiation is inhibited. In order to assess a possible involvement of Pref-1 in this blockage an RT-PCR assay was carried out with these clones. However, after amplification, the 449 bp fragment was seen in all samples analysed even for those that differentiated into adipocytes including the control (Figure 8.8 E). The reason for this might be due to the fact that after differentiation into adipocytes there is always a population (~5-10%) of cells that do not differentiate and remain as preadipocytes. Thus, due to the high sensitivity of the PCR reaction just a small number of template molecules may be enough to give a positive signal. This

problem has been observed by other groups and a purification step of differentiated adipocytes from the remaining preadipocytes by differential centrifugation with bromobenzene has been suggested to overcome it (Smas and Sul, 1993).

Several studies have shown that the inhibition of cell division of cultured preadipocytes lines clearly blocks their subsequent differentiation. In order to assess whether cell division was impaired in these clones transfected with RAIDD, it was decided to study the proliferation rate of these cells thorough the different steps of the differentiation program. The same number of cells from control parental 3T3L1 and one of the clones transfected with RAIDD that had shown blockage of the differentiation were plated on 6 well dishes and the number of cells determined with an hematocytometer at different stages of the differentiation program, including at confluence (-2), after induction with the hormones (+2) and after 6 days of differentiation (+6).

Figure 8.9 shows similar number of cells for both groups when they reached confluence thus indicating no inhibition in the proliferation rate for this clone transfected with RAIDD in the preadipocyte state. Furthermore, after induction to differentiate with the cocktail of hormones (+2 in Figure 8.9) both groups increased the number of cells according to the clonal expansion induced by the combination of mitogenic and adipogenic signals provided in the differentiation medium. Finally, after 6 days of differentiation the number of cells remained similar for both groups, although parental 3T3L1 cells underwent conversion into adipocytes, while this clone transfected with RAIDD remained as preadipocyte. These results suggest that cell proliferation is not impaired in these clones transfected with RAIDD and this should not be the cause of the blockage of these cells to differentiate.

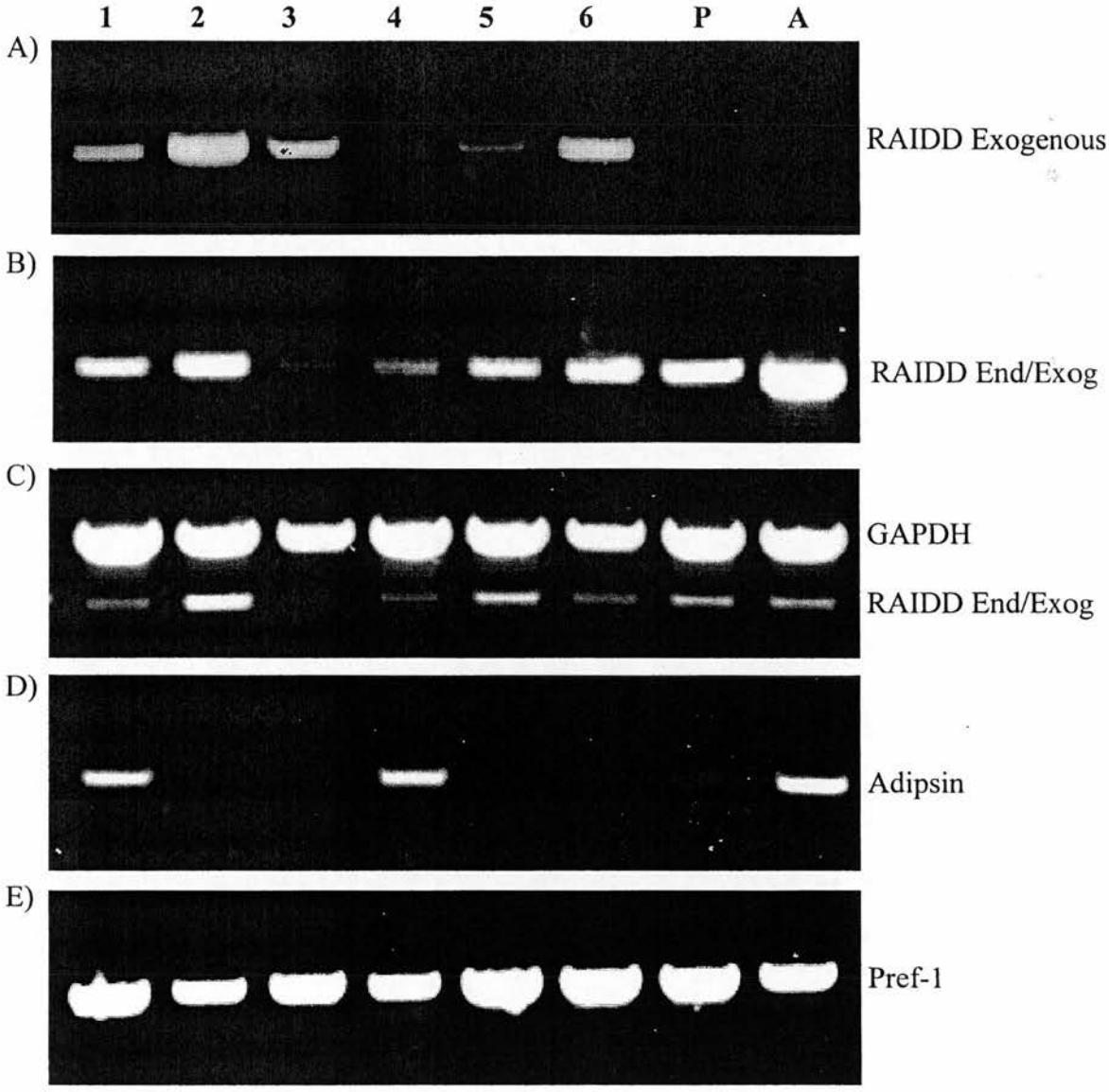


**Figure 8.8 Expression of various mRNAs from cells transfected with the murine RAIDD cDNA.**

5 μg of total RNA was DNAse treated, reverse transcribed with oligo (dT)12-18 mer (Gibco BRL) and subjected to PCR with primers for the different genes as described in the figures.

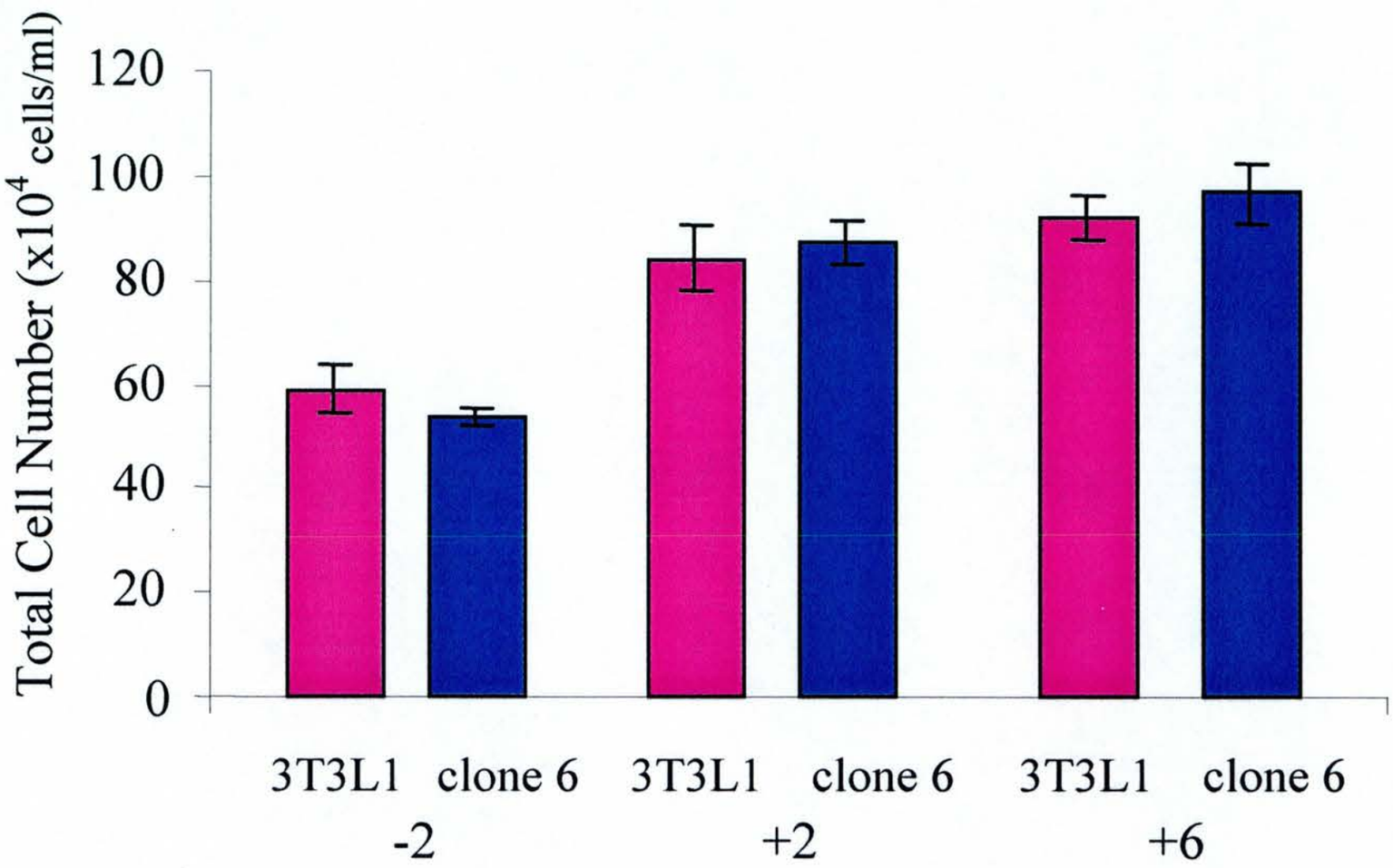


**Figure 8.9 Cell number quantitation at different points of the differentiation program.**

Cell number of the parental cells (3T3L1) and one of the clones transfected with RAIDD (clone 6) were counted at different points of the differentiation program including at confluence (-2) after hormonal induction (+2) and after 6 days of differentiation (+6).

Each bar represents the mean ± SD of duplicates.

These studies allowed to conclude that adipogenesis as measured by i) lipid accumulation, ii) cell morphology, and iii) induction of adipocytes-specific marker proteins is severely inhibited in those clones transfected with RAIDD. These findings indicate that an overexpression of RAIDD during adipocyte differentiation is sufficient to fully inhibit 3T3L1 differentiation. Enforced expression of RAIDD could block cell differentiation by perturbing one or several events that are important steps leading to the terminally differentiated state.

## 8.5 REGULATION OF TRANSCRIPTION FACTORS C/EBPα AND C/EBPβ EXPRESSION DURING ADIPOCYTE DIFFERENTIATION OF CLONES TRANSFECTED WITH THE MURINE RAIDD cDNA.

Adipocyte growth and differentiation follow patterns of sequential B-ZIP protein expression, including AP-1 (Distel *et al.* 1987) and C/EBP family proteins (McKnight *et al.* 1989). C/EBPα is the first member of the C/EBP family discovered (Birkenmeier *et al.* 1989). It is expressed at high levels in both white and brown adipose tissue of rodents and accumulates during adipocyte differentiation with a temporal pattern similar to the acquisition of the differentiated phenotype. C/EBPα is expressed just before the activation of several adipocytes specific genes, a number of which contain C/EBP binding sites in their promoter (Herrera *et al.* 1989). Some of these genes include glycerophosphate dehydrogenase, fatty acid synthase, acetyl CoA carboxylase, Glut 4, insulin receptor and aP2, the adipocyte-selective fatty acid binding protein (Spiegelman *et al.* 1993). Definitive proof that C/EBPα is required for adipocyte differentiation came from the demonstration that antisense inhibition of C/EBPα inhibited the terminal differentiation of 3T3L1 cells (Lin and Lane, 1992). Consistent with this finding, knockout mice lacking C/EBPα die shortly after birth with no discernible development of white adipose tissue (Wang *et al.* 1995). Another possible function suggested for C/EBPα in the differentiation is maintenance of the differentiated state through autoactivation of its own gene whose promoter contains C/EBP binding sites (Lin *et al.* 1993).

Another C/EBP gene (C/EBPβ) which, was discovered later than the C/EBPα (Cao *et al.* 1991), functions prior to C/EBPα as transcriptional activator and it is transiently induced by hormones such as cAMP or agents that increased its levels as methylisobutylxanthine used in the differentiation program, though the induction by these external hormones is just transient since expression is also observed in actively dividing preadipocytes (Cao *et al.* 1991). The double knockout of C/EBPβ and C/EBPδ, another member the C/EBP family also results in reduced

fat mass (Tanaka *et al.* 1997), all this suggesting that C/EBP family proteins are crucial factors in fat development.

Having established an initial characterization of the blockage in differentiation of clones of 3T3L1 transfected with RAIDD, it was decided to study in more detail the levels of expression by Northern blot of some of these crucial transcription factors involved in the differentiation of 3T3L1 into adipocytes. It was thought that RAIDD might be interfering in the normal expression of these transcription factors thus precluding these cells from differentiating. 3T3L1 preadipocytes were induced to differentiate by using previously established methods as described in chapter 2 and above (Student *et al.* 1980). Cells were grown to confluence in growth medium containing calf serum and after two days postconfluence shifted to differentiation medium containing 10% fetal calf serum, insulin, dexamethasone and methylisobutylxanthine. Two days later, dexamethasone and methylisobutylxanthine were removed. The progress of the differentiation was monitored by measuring the accumulation of RNA encoding for the adipocyte-specific protein aP2 (Bernlohr *et al.* 1984). Total RNA was harvested from cells immediately before and at intervals after the addition of the differentiation medium.

The Northern blot in Figure 8.11 shows that expression of the mRNA encoding fatty acid binding-protein aP2 was first detectable at low levels after the induction to differentiate (day 3) and rose to maximal levels by day 8 in the control cells but failed to appear in the other two clones. Only clone 6 showed a very low signal after 8 days of differentiation thus confirming the failure of these cells to fully express the differentiation markers and to accumulate triglycerides. Expression of aP2 was accompanied by changes in cell morphology of the controls that included the accumulation of triglycerides droplets in the cytoplasm of the cells (not shown). This effect was not observed in any of the two clones analysed in this experiment.

The RNA samples collected before and during differentiation were also hybridised with radioactively labeled cDNA fragments (purchased from the UK HGMP Resource Centre) corresponding to C/EBPα and C/EBPβ genes. Consistent

with earlier studies (Birkenmeier *et al.* 1989), the mRNA encoding for C/EBPα was not detected previous to the differentiation and was first detected at low levels on day 2 and increased at day 8 in the control cells. However, no signal was observed in any of the stages for the two clones analysed. The mRNA encoding for C/EBPβ on the other hand, was detectable in all undifferentiated cells and remained detectable during the latter half of the differentiation program consistent with earlier studies carried out by Cao *et al.* (1991). These findings strongly suggest that the blockage in the differentiation of these clones correlates with the inappropriate expression of the transcription factor C/EBPα.

Induction of C/EBPβ and C/EBPδ expression is thought to be necessary for the subsequent induction of C/EBPα and PPARγ[2], which in turn act to coordinate the expression of a number of fat specific genes required for lipid metabolism, including aP2, SCD1, and Glut 4. In marked contrast to C/EBPβ, the expression of C/EBPα was undetectable in the two clones analysed but was detectable in the control cells. These results confirm the important role of C/EBPα in lipogenesis. Furthermore, the expression of the adipocyte specific marker aP2 was severely inhibited or undetectable in these clones thus demonstrating an impaired expression of the transactivating protein C/EBPα.

These observations provide a molecular mechanism for the inability of the cells transfected with RAIDD to accumulate cytoplasmic triglycerides and to differentiate into adipocytes. Figure 8.10 below shows a summary of the stages where RAIDD might be interfering in the appropriate differentiation of these clones.

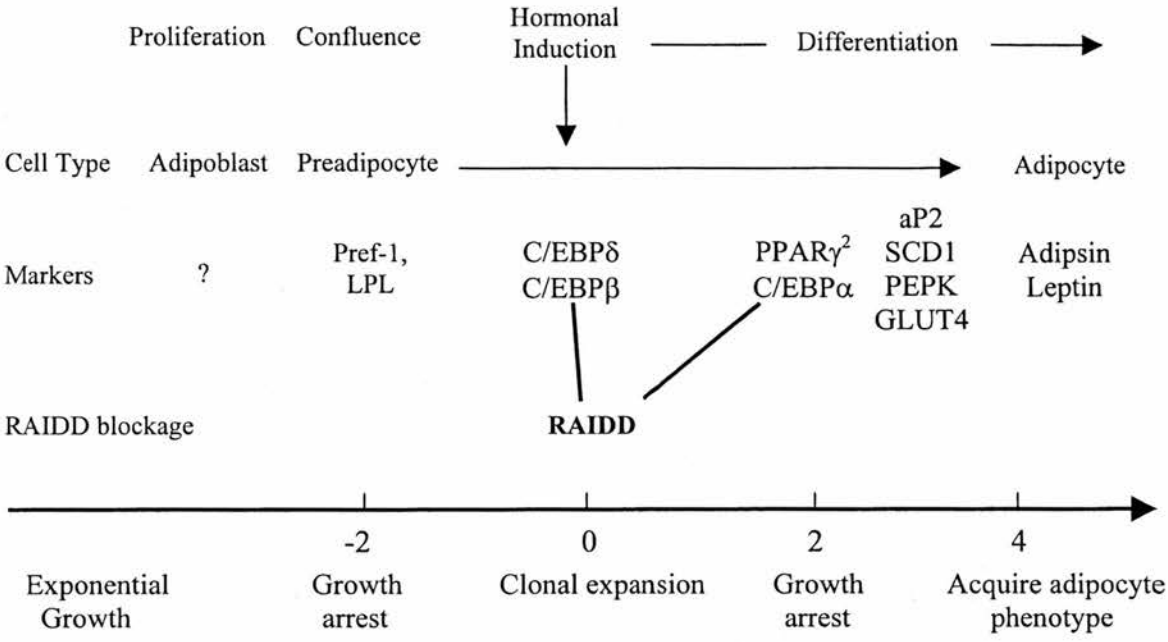


**Figure 8.10 Model of RAIDD inhibitory effect in the adipocyte developmental program.**

RAIDD inhibitory effect is specifically in the terminal stages of the differentiation. RAIDD may interfere with the activation of C/EBPα by inhibiting the transactivation function of C/EBPβ (or C/EBPδ) or competing with the C/EBP family of proteins to bind to their specific DNA target sequences in the C/EBPα promoter (or PPARγ2).

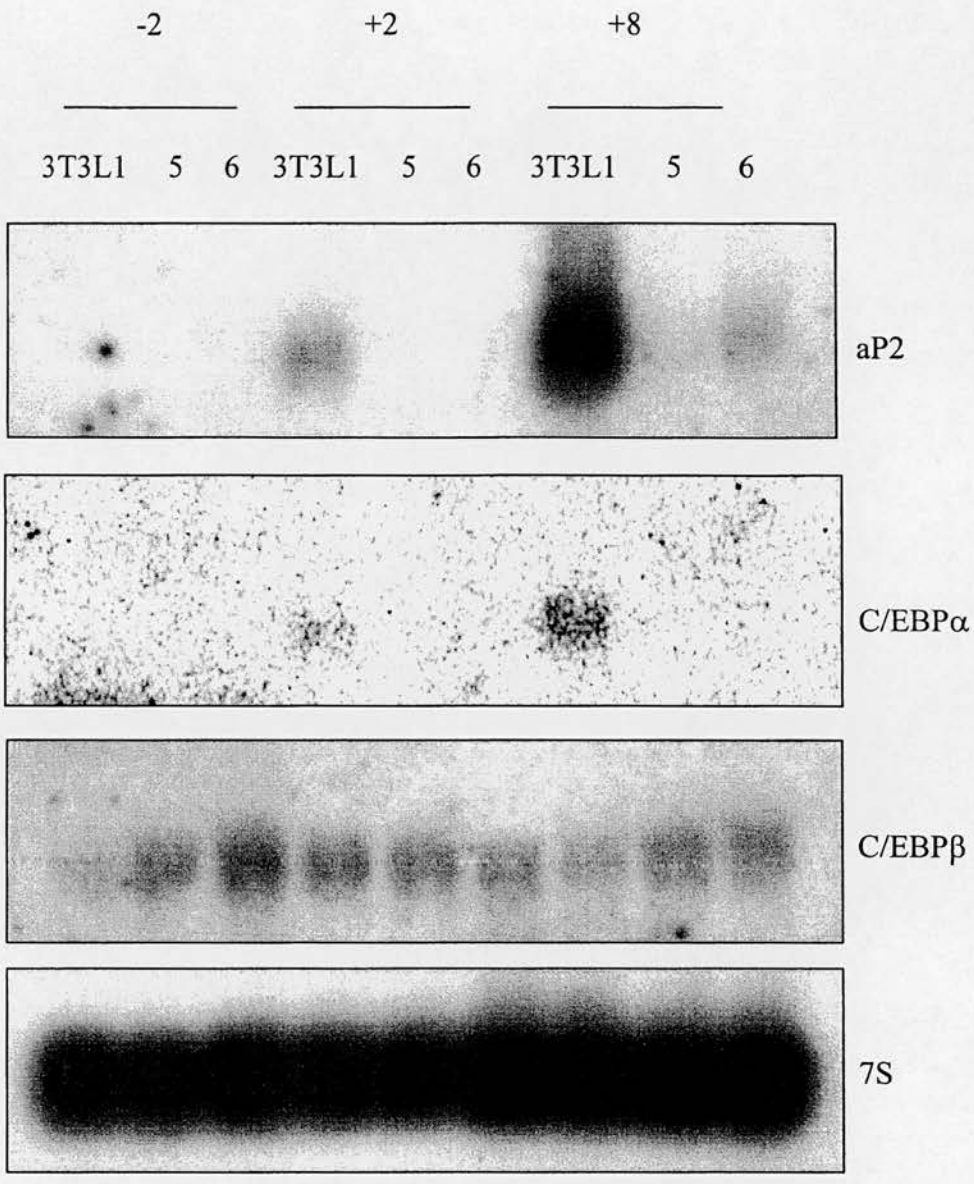


**Figure 8.11 Northern analysis to assess the expression of crucial transcription factors in the differentiation program of 3T3L1 cells.**
10 μg of total RNA from two clones transfected with RAIDD and the parental cells 3T3L1 after different days of the differentiation program were run on a denaturing agarose gel and Northern blot hybridised with probes: aP2, C/EBPα, C/EBPβ and 7S as loading control.

## 8.6 CONCLUSION.

Differentiation of 3T31 cells transfected with the murine RAIDD cDNA resulted in a range of phenotypes from which the most surprising was a complete blockage of these cells to differentiate into adipocytes, after exposure to the cocktail of hormones/inducers. Other clones showed a partial accumulation of triglycerides or differentiated adipocytes that escaped the blockage begun to die. These findings suggest a possible dose response effect of the expression of RAIDD during differentiation of these cells. A possible scenario for these effects would be that higher levels of expression of RAIDD early in the differentiation would result in the blockage of these cells to differentiate. On the other hand, lower levels of expression early in the differentiation would allow these cells to proceed normally through the differentiation program but once aP2 promoter reaches its maximum activity (after 10 days of the differentiation, see Figure 3.10 in chapter 3) higher levels of RAIDD expressed from this promoter would render these cells susceptible to the RAIDD-mediated ablation. Although the aP2 promoter does not support expression in preadipocytes (Figure 3.10 in chapter 3), and its maximum activity is reached only after ~10 days of the differentiation program, one might expect certain variation in the developmental expression of a transgene driven by this promoter, if the transgene is inserted in a site in the chromosome which is more permissible for its expression. Furthermore, Lin and Lane (unpublished observations) have observed an extremely low level of aP2 mRNA by RNase protection analysis in confluent, but not proliferating, 3T3L1 preadipocytes before differentiation, thus confirming that expression from this promoter can be observed earlier in the differentiation program of these cells even before transactivation from C/EBPα.

Enforced expression of RAIDD could block cell differentiation by perturbing one or several events that are important steps leading to the terminally differentiated state. One can speculate on both positive (stimulation of anti-differentiation genes) and negative (interference with expression of differentiation genes) mechanisms. By analysing patterns of gene expression characteristic of each phase of the differentiation program, it was determined that the RAIDD-mediated block in differentiation of 3T3L1 is manifested during the terminal stages of the

differentiation at least in those clones that failed to differentiate, since it was excluded the alteration in the expression of the early stage gene C/EBPβ. The more likely scenario is that a decrease in the expression of prodifferentiation proteins such as C/EBPα and possibly PPARγ (not analysed) prevent transactivation of the down regulated adipocyte specific genes. Consistent with this view are the experiments that demonstrated inhibition of the differentiation of 3T3L1 with antisense C/EBPα (Line and Lane, 1992). Since C/EBPα and PPARγ2 are thought to act in concert to induce expression of genes such as aP2, SCD1 which are required for lipid metabolism, these results provide a molecular mechanism for the inability of these cells transfected with RAIDD to accumulate triglycerides.

While these studies (may) define a specific point at which RAIDD represses adipogenesis, they do not in themselves provide any mechanistic explanation of how this happens. Since RAIDD function is primarily believed as an adapter in the apoptotic-signalling pathway, it is possible that RAIDD differentiation block occurs indirectly acting in a negative manner to block the transcription of one or more of these masters genes as the results already shown suggest.

Whatever the mechanism underlying the blockage effect mediated by RAIDD, the results presented here encourage further studies especially in a mouse model overexpressing RAIDD in the adipose tissue. One might speculate that no matter the result (adipocyte blockage or adipocyte death) the potential mouse model that could be generated may provide an alternative system to ablate adipocytes and a novel model where to study fat metabolism and the contribution of adipose tissue to the pathogenesis of obesity and diabetes. Alternatively, this system could also provide a new approach to the ablation of other cell types, thus allowing the creation of different animal models for human diseases.

## 9. OVERVIEW.

Adipocytes play a central role in maintaining energy balance in animals, storing energy in the form of triglycerides in periods of nutritional abundance and releasing it in a metabolically useful form at times of nutritional deprivation. The inability of investigators to direct expression specifically to adipose tissue was a major problem in the use of transgenic technology to study this tissue. The discovery of a regulatory element (Ross *et al.* 1990) that can direct tissue specific expression to adipocytes has opened up the possibility of experiments to regulating the fatness of laboratory and domestic animals and the study of the metabolism and physiology of this tissue. Some of these studies have included the delivery of toxic genes to the adipose tissue (Ross *et al.* 1993, Burant *et al.* 1997), expression of transgenes that can alter the amount or distribution of body fat by influencing energy balance, or disrupting adipocyte differentiation or proliferation (Soloveva *et al.* 1997, Moitra *et al.* 1998, Shimomura *et al.* 1998) and finally the delivery of transgenes that are non-toxic themselves but that can be activated by the downstream administration of a substance (prodrug) that make them capable of selective cell killing once they are activated as it is the NTR/CB1954 system (Clark *et al.* 1997) that was used in this research project.

In the experiments more relevant to this thesis, the aP2 promoter/enhancer was used to drive the expression of the Diphtheria toxin A chain to the adipose tissue. High levels of expression caused neonatal death, while lower levels produced loss of fat in aging mice and a resistance to diet induced obesity (Ross *et al.* 1993; Burant *et al.* 1997). These results suggested that white adipose tissue was an essential organ for life since no mouse model from either knockout or transgenic technology had been created that was devoid of fat throughout development. Nevertheless, two teams that used the same tissue-specific promoter recently challenged this view creating WAT-free laboratory mice. While Shimomura *et al.* (1998) directed adipocyte specific expression of a truncated form of SREBP1c, a transcription factor involved in biosynthesis of both cholesterol and fatty acids,

Moitra *et al.* (1998) on the other hand, utilised a truncated dominant negative transcription factor that would bind and inhibits the function of transcription factors that are critical for fat development. The resulting transgenic mice were remarkably similar, WAT-free, profoundly insulin resistant, and markedly diabetic. This came as a surprise since the general perception was that diabetes was a disease of the obese. As important as these results are they fail in one simplistic but important thing which is that they do not have the control over the ablation of the adipose tissue, something that would be possible with an inducible system such as the NTR/CB1954 used in this thesis. Experiments in transgenic animals using other gene suicide systems have shown that targeted ablation of specific cell types in the context of the whole animal can be a powerful tool for the study of organ development and function and for the generation of transgenic models of human diseases (Palmiter *et al.* 1987; Behringer *et al.* 1988; Bernstein and Breitman, 1989).

One potential problem associated with the use of some of these suicide systems such as Diphtheria toxin A, or Ricin A derives from its extreme cytotoxicity. Although this ensures that cells expressing low levels in a targeted cell population will be killed, it also means that any leakiness from the transcriptional regulatory elements that may result in ectopic expression of the toxin could lead to embryo lethality or deleterious effects that could compromise the analysis of the resulting transgenic animal. Ectopic expression of the targeting promoter sequences could pose a major problem, especially if exacerbated by chromosomal position effects, which are known to results in variable levels of expression among randomly integrated transgenes (Palmiter and Brinster, 1986; Dobie *et al.* 1996). Therefore, inducible ablation with NTR/CB1954 system affords the practical advantage of allowing stable pedigrees to be established before the initiation of the ablation studies. It also provides the means to control the timing and duration of toxic insult and to assess the potential for recovery of an ablated cell population after a prodrug free period in the transgenic animal.

When compared with other inducible systems such as *tk*/GCV, NTR/CB1954 still provides some important advantages that make this system a good alternative for the ablation of specific cell populations in a transgenic model. While conditional ablation with *tk* gene is likely to be effective only in proliferating cells, as cell

killing is mediated through inhibition of DNA synthesis (Borrely *et al.* 1989) many potential target cell populations, such as non-cycling stem cells and terminally differentiated cell types such as adipocytes or neurons cells are likely to be refractory to the toxic effects of the administered drug, despite significant expression of the herpes *tk* gene. In addition to this, CB1954 readily crosses the blood-brain barrier thus making feasible to target specific cell types within the central nervous system (CNS). Furthermore, CB1954 is readily administered by intraperitoneal injection whereas most ablation studies using *tk*/GCV have used minipumps.

The work presented in this thesis was undertaken as a first step to assess the susceptibility of non-dividing cells, such as adipocytes, to the ablation by this novel approach NTR/CB1954 in the intact animal. To target the expression of the *E.coli* nitroreductase gene into the adipose tissue, regulatory elements from the adipocyte-specific lipid biding protein gene (aP2) were used (Ross *et al.* 1990). Preliminary experiments in cells showed that this 5.4 kb fragment targets expression of linked genes in a developmental manner similar to endogenous aP2. Activation of the transgene by this promoter sequence indicated that *E.coli* NTR is not detrimental for the survival of the cells and more importantly for an appropriate differentiation of these cells into adipocytes. In the same way, no side effects associated with carrying or expressing the NTR transgene were observed in those lines of transgenic mice generated by microinjection and both sexes were fully fertile. This data confirmed that the sequences of the aP2 promoter used contain all the necessary elements to drive relatively strong levels of expression both *in vitro* and *in vivo*. The demonstration of the appropriate translation of this prokaryotic protein confirmed that eukaryotic cells (adipocytes) have no major problems in expressing this E.*coli* NTR sequence.

When differentiated adipocytes were challenged to CB1954, specific killing of nitroreductase expressing cells was observed. The degree of cytotoxicity correlated with the level of nitroreductase activity in these cells, presumably due to a more efficient conversion of the prodrug to the active metabolites, in agreement with previous work in murine fibroblast and other human tumour cell lines (Bridgewater *et al.* 1995; Green *et al.* 1997; McNeish *et al.* 1998). In the same way, low

concentration of the prodrug rendered these cells more resistant to the killing, requiring longer exposure times to achieve a similar toxic effect. Presumably at lower concentrations the cells are still capable of repairing the DNA adducts formed as a result of the bioactivation of the prodrug and over certain concentration they are no longer capable of doing so. As a consequence of these lesions in the DNA, it has been proposed that a self-destructive mechanism known as apoptosis is activated in these cells. Experiments described in chapter 3 clearly confirmed that adipocytes stably expressing the *ntr* gene die, after administration of CB1954, by this mechanism of apoptosis. Both morphological and cellular data are consistent with this observation. TdT-mediated dUTP nick end labeling (Tunel) showed specific staining indicative of apoptosis-mediated DNA strand breaks in cells expressing NTR treated with the prodrug. Furthermore, DNA fragmentation and morphological analysis confirmed the apoptosis-mediated cell death.

Treatment of transgenic mice with the prodrug also showed specific killing of adipocytes whereas other tissues were normal and comparable with non-transgenic treated or untreated control mice, confirming the efficacy of this system to kill terminally differentiated (non-dividing) cells in an animal model. Furthermore, histological analysis of these mice showed that ablation of adipocytes was not restricted to an specific fat pad since all major adipose deposits analysed showed reduction or disappearance of adipose cells. As in the *in vitro* studies, the magnitude of cell ablation was directly correlated to the dose of CB1954 used with a much stronger effect at doses of 40 mg/kg of body weight. This higher dose resulted in the complete disappearance of any visible adipose deposits in some transgenic mice, thus suggesting that a bystander effect may have occurred *in vivo*, since immunohistochemical analysis of sections of adipose tissue confirmed variegated transgene expression in these cells.

Variegated transgene expression is a common problem in transgenesis. This phenomenon results in patchy expression, frequently observed as cluster of expressing cells next to clusters of non-expressing cells. The reason for this is not clear but it has been suggested that the chromosomal site of transgene integration and the transgene copy number may be responsible (Dobie *et al.* 1996). Thus, transgenes inserted close to the centromere are more prone to random inactivation.

This has been observed with many transgenes and it would appear that adipose tissue is particularly prone to variegated expression since both lines analysed in this thesis (lines 30 and 87) showed this problem. This mosaic expression was shown to have important implications in the proposed ablation experiments, since it prevented the ablation of 100% of adipocytes and resulted in a variable number of remaining cells in this tissue. Nevertheless, complete disappearance of any visible fat pad could also be observed in some experiments especially with higher doses of the prodrug, which suggests that *in vivo* bystander effect of the prodrug may have occurred.

A number of studies attempting gene delivery to tumours has shown that an important proportion of cells remain unmodified (Ram *et al.* 1993). Gene delivery is probably limited both by the efficiency of various current gene delivery technologies and by restricted access to some tumour regions. Thus, a desirable feature of enzyme/prodrug combinations for use in gene directed enzyme prodrug therapies (GDEPT) is that the killing of modified cells result in the killing of adjacent unmodified cells. This bystander effect, originally described with *tk*/GCV system (Freeman *et al.* 1993), had been previously observed with the NTR/CB1954 by some authors (Knox *et al.* 1988; Bridgewater *et al.* 1995), although the same effect could not be observed by others (Clark *et al.* 1997). Here, *in vitro* studies clearly confirmed this bystander effect of CB1954 in adipocytes and it was demonstrated that this effect is due to a toxic metabolite of CB1954. A similar bystander effect could be suggested to occur *in vivo*. The fact that this bystander effect has not been always observed with this system can be attributed to the intrinsic resistance of some cells to the prodrug or the lack of intimate cellular contact between nitroreductase expressing and non-expressing cells. The short half-life of the active metabolites is another possibility. This bystander effect is especially useful in gene addition experiments involving the use of toxigenes, such as the experiments described in this thesis, where the effects of variegated expression might silence the expression of the transgenes. Therefore, this bystander effect will enable the killing of surrounding silenced cells. On the other hand, the lack of a bystander effect, as observed in other studies should also encourage the belief that the ablation of specific cells in tissues such as pituitary, pancreas, CNS and immune system, which

comprise more than one cell type in close association, can be accomplished with this approach. This would allow cell-lineage studies to unravel the developmental relationships between different cells in a tissue. Clearly what may be a desirable attribute in one set of circumstances need not be so desirable under other circumstances. Thus in cell-lineage studies the aim might be to minimise the bystander effect whereas in tumour destruction the aim would be to maximise the response.

The lack of adipocytes led to dramatic consequences in some transgenic mice especially in their ability to thermoregulate their body temperature. More extreme cases included the death of some mice confirming the importance of the adipose tissue for the normal development of life. Treatment of transgenic mice with a lower dose of CB1954 results in a partial reduction of the adipose tissue leaving remaining cells that do not express the transgene and thus escape the ablation with the prodrug. These mice show no problems to thermoregulate body temperature and therefore can be used for studies related to the consequences of fat ablation. One of these studies assessed the ability of this tissue to regenerate after a prodrug free period. This observation is based on the analyses of the total body lipid content in the carcasses of these mice and the measurements of the wet weight of the gonadal fat pads, both of which showed compensatory growth after a prodrug free period. Interestingly, it was found that the compensatory growth of the adipose tissue did not include newly formed transgenic cells. The reason for this is not clear but a possible scenario might be that preadipocytes or stem cells die as result of a bystander effect. It is believed that the mechanism of regeneration of the adipose tissue might involve hyperplasia and/or hypertrophy of the remaining cells. This observation is based on circumstantial evidence from immunohistochemistry analyses that showed a number of small adipocytes growing alongside the much larger mature fat cells. Confirmation of this should come from analysis of the number and size of these cells in each of the fat pads.

Partial reduction of adipocytes caused no major physiological consequence, with blood glucose, and plasma triglycerides, FFA and β-hydroxybutyrate levels remaining normal or at levels comparable to the control groups. Nevertheless, analysis of leptin showed that this hormone decreased significantly after ablation of

adipose tissue thus, corroborating the proposed role of this hormone as an adiposity indicator, the levels of which return to normal after a recovery period of 30 days.

It is necessary to stress that a different situation might have been expected with complete penetrance of the transgene i.e. if 100% of the cells had expressed the transgene. In such situation one might expect that treatment with the prodrug should enable the killing of nearly all adipocytes in the fat pad the consequences of which could be as dramatic as those observed in clinical patients suffering of the genetic condition known as lipoatrophic diabetes. Similar effects to those observed in these patients were observed in two mouse models devoid of adipose tissue (Shimomura *et al.* 1998, Moitra *et al.* 1998). Despite the lack of fat, these mice developed diabetes, a condition normally associated with excess rather than a decrease in the adipose tissue.

It would be interesting to test the effect of the prodrug in the other transgenic lines that were generated. It is possible that these lines have a more uniform expression in the adipose tissue. Immunohistochemical analysis should clarify this. Alternatively, the creation of transgenic mice expressing the NTR by means of gene targeting in ES cells might provide a better option to ensure the incorporation of single copies of the transgene in a permissible site in the chromosome, which might be less prone to effects of variegated transgene expression.

The inducible ablation of adipocytes in this transgenic model should provide unique opportunities to study the effects of decreasing the number of adipocytes at different stages of the mouse development including juvenile, adult and in pathological conditions such as obesity, thus allowing the investigation of the interrelationship between excessive adipose tissue mass and the associated disorders (diabetes, hypertension, hyperlipidemia) through manipulation of the target cell population. It is precisely obesity one of the diseases that could be best approached with this model. The phenotype of the obese mice is consistent with the hypothesis that metabolic abnormalities in diabetes are the result of an excess of adipose mass. Although the preliminary experiments shown in chapter 7 are not very informative with respect to the real potential of this approach, these must be taken as the starting point for future experiments in this field. Conditional ablation in this model would allow the removal of adipocytes before the symptoms of diabetes are manifested,

therefore making it possible to assess the link between the excess of adipose tissue and the associated disorders. To avoid long breeding generations with the obese mice, and to avoid the effects of the different genetic background it would be possible to induce obesity by overfeeding one of the wild-type mouse expressing the NTR in the adipose tissue. Alternatively, obesity could be induced by chemical damage in the hypothalamus (Bray and York, 1979, Ross *et al.* 1993) such as with the administration of monosodium glutamate at birth in one of these transgenic mice. More straightforward questions that could be answered with this model include the link between infertility and excess of adipose tissue. A number of studies have shown that reduction of adipose tissue mass restore fertility (Ross *et al.* 1993), therefore it would appear that infertility in obese mice results from an excess of adipose tissue. It has been suggested that this could be the result of exogenous steroid production by adipose tissue, resulting in high estrogens or androgen levels (Foster DW, 1985). Although it has been shown that leptin treatment, but not food restriction, can rescue the infertility of *ob/ob* mice, it would be interesting to assess whether reducing the adipose tissue would also restore the fertility of these mice.

To further expand the wide spectrum of applications of this inducible system of NTR/CB1954, *in utero* ablation experiments were carried out to assess the feasibility of the prodrug to cross the transplacental membrane. Experiments shown in chapter 7 confirmed the ability of CB1954 to cross the transplacental barrier and more importantly to induce ablation of nitroreductase expressing cells in the developing pups. CB1954 produced a decreased in the number of transgenic pups generated, which suggests a direct effect of the prodrug in those mice expressing the NTR in the brown adipose tissue. Although is not clear whether transgenic offspring died because of their inability to thermoregulate, two transgenic pups that survived showed a large reduction in the size of the brown adipose tissue. It has been long ago suggested that increased BAT thermogenic activity could prevent obesity when a high caloric diet is given to mice and rats (Rotwell and Stock, 1979). Although this hypothesis was based only in circumstantial evidence, it was not until Lowell *et al.* (1993) created transgenic mice that became obese because of a large reduction in brown adipose tissue, that this hypothesis was proved right. The possibility to ablate brown adipose tissue with NTR/CB1954 could then provide an alternative model

where to study the consequences of the ablation of this tissue. These experiments encourage the belief that ablation of embryonic cells and other fetal cells can be accomplished with this approach, which expand substantially the spectrum of opportunities of this system.

Finally, the last chapter of this thesis described the preliminary experiments carried out with a preadipocyte cell line (3T3L1) overexpressing the murine RAIDD cDNA in an attempt to test the feasibility of the RAIDD-mediated ablation in these cells. A range of phenotypes was observed in clones exposed to the cocktail of hormones that induce the conversion of these cells into adipocytes. The most notorious effect was a complete blockage of the differentiation of these cells into adipocytes. Enforced expression of RAIDD could block cell differentiation by perturbing one or several events that are important steps leading to the terminally differentiated state. One can speculate on both positive (stimulation of anti-differentiation genes) and negative (interference with expression of differentiation genes) mechanisms. Since RAIDD function is primarily believed to be an adapter in the apoptotic-signalling pathway, it is possible that the RAIDD differentiation block occurs indirectly, acting in a negative manner to block the transcription of one or more of these masters genes. Whatever the mechanism underlying the blockage effect mediated by RAIDD, these results encourage the belief that it would be possible to use this system as a novel approach to reduce adiposity in mice. One can speculate that no matter the result (adipocyte blockage or adipocyte death) the potential mouse model that could be generated would provide an alternative system where to study fat metabolism and the contribution of adipose tissue to the pathogenesis of obesity and diabetes.

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